

ROLE OF RETINOIC ACID IN ACUTE LUNG INFLAMMATION AND NEUTROPHIL
BIOLOGY

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By

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ABSTRACT

Acute lung injury is a severe clinical condition with high mortality both in animals and human beings. It is characterized by increased vascular permeability, accumulation of activated neutrophils, and diffuse damage to lung parenchyma. Neutrophil-derived toxic metabolites play a pivotal role in pathogenesis of acute lung injury. One of the ways to ameliorate acute lung injury is to modulate neutrophil functions. Retinoids are a group of compounds that include natural vitamin A and its synthetic derivatives. Role of retinoids in modulation of inflammatory and immune response is well established by previous studies. Effects of retinoids are mediated through two families of nuclear receptors; retinoic acid receptors (RAR) and retinoid X receptors (RXR). There are three subtypes in each family, namely; α , β , and γ . There are limited data available on the species-specific expression of various retinoid receptors and the role of retinoic acid in acute lung inflammation. Therefore, I designed a series of studies to evaluate the expression of retinoid receptors in many species and the role of retinoic acid in neutrophil apoptosis and acute lung inflammation.

First, I determined the expression of retinoid receptors in intact lungs of cattle (n=2), pigs (n=2), dogs (n=2), mice (n=5), and humans (n=3). Normal and inflamed mouse lungs (n=5/group), normal human lungs (n=3) and inflamed human lungs (n=3) from patients who died of sepsis were also used. The expression was determined with multiple methods such as western blots, immunohistology, and immunoelectron microscopy. Normal lungs from all of the species showed differential expression of retinoid receptor subtypes in airway epithelium, vascular endothelium, alveolar/septal macrophages, and alveolar septum. In cattle pulmonary intravascular macrophages also showed expression of retinoid receptor subtypes. Intranasal treatment with *E. coli* lipopolysaccharide (LPS, 055:B5, 80 μ g) resulted in increased expression of RXR α in airway epithelium of mouse lungs.

Having examined the expression of retinoid receptors, I determined the effects of retinoic acid on spontaneous apoptosis of canine neutrophils using flow cytometry, caspase-3 assay, light microscopy, Terminal Transferase dUTP Nick End Labeling (TUNEL) Assay, and electron microscopy. Neutrophils were treated either with *E. coli* LPS (1 μ g/ml) alone or with both LPS and retinoic acid (1 and 5 μ M) for 12 hours and 36 hours. Treatment with LPS resulted in reduced number of apoptotic neutrophils compared to untreated cells ($p < 0.05$). Simultaneous treatment with both LPS and retinoic acid abolished this effect. This suggests that retinoic acid reverses LPS-induced delay in spontaneous apoptosis of neutrophils.

After studying the effects of retinoic acid on canine neutrophil apoptosis, I used a mouse model of acute lung injury to investigate the effects of retinoic acid on endotoxin-induced acute lung inflammation. Intranasal LPS treatment resulted in robust infiltration of neutrophils into the alveoli. Retinoic acid pre-treatment (10 mg/kg) 30 minutes before LPS treatment resulted in the reduction of total cell counts ($p < 0.05$) and percentage of neutrophils in bronchoalveolar lavage (BAL) fluid at 6 hours after the LPS treatment. Protein content in BAL fluid was also decreased ($p < 0.05$) following pre-treatment with retinoic acid. Lung tissues showed significantly reduced numbers of myeloperoxidase-positive cells in LPS-treated mice pre-treated with retinoic acid. However, retinoic acid had no effect on cytokine and chemokine levels (TNF- α , IL-1 β , IL-10 and KC) in BAL fluid. Effect of retinoic acid on canine neutrophil chemotaxis was also studied under *in vitro* conditions. Activation with *E. coli* LPS (1 μ g/ml) induced chemotaxis of neutrophils towards recombinant human interleukin-8 ($p < 0.05$). However, retinoic acid did not exert inhibitory effects on the LPS-induced chemotaxis of canine neutrophils.

Taken together, the data from these studies show that retinoid receptors are expressed in normal and inflamed lungs, and treatment with retinoic acid hastens apoptosis in activated neutrophils and inhibits neutrophil recruitment and permeability in lipopolysaccharide-induced acute lung inflammation.

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LIST OF ABBREVIATIONS

ALI	Acute lung inflammation
AM	Alveolar macrophages
ANOVA	Analysis of variance
AP-1	Activator protein-1
ARDS	Acute respiratory distress syndrome
ATRA	All- <i>trans</i> retinoic acid
BAL	Bronchiolar alveolar lavage
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CRBP	Cellular retinol binding protein
DBD	DNA binding domain
EC	Endothelial cell
EM	Electronmicroscopy
fMLP	N-formyl-methionine-leucine-phenylalanine peptide
GM-CSF	Granulocyte macrophage colony stimulating factor
G-CSF	Granulocyte colony stimulating factor
HATs	Histone acetylase
IL	Interleukin
ICAM	Intercellular adhesion molecule
KC	Keratinocyte chemoattractant
LBD	Ligand binding domain
LPS	Lipopolysaccharide

MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinases
NF- κ B	Nuclear Factor-KappaB
PECAM	Platelet endothelial cell adhesion molecule
PIK3	Phosphatidylinositol 3-kinase
PKA	Protein Kinase A
PKC	Protein kinase C
RA	Retinoic acid
RAR	Retinoic acid receptor
RBP	Retinol-binding protein
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SRC	Steroid receptor co-activator
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
VCAM	Vascular cell adhesion molecule
vWF	von Willebrand Factor

CHAPTER1: REVIEW OF LITERATURE

1.1.Introduction

Acute lung inflammation is a severe clinical condition with high morbidity and mortality. Despite significant progress in understanding pathogenesis and therapeutic interventions, the mortality rate in acute lung injury still remains up to 40% (Ware and Matthay 2000). It is characterized by increased expression of adhesion molecules, accumulation of activated neutrophils, pulmonary edema, and diffuse damage to lung parenchyma. Pathogenesis of acute lung injury is complex and poorly understood. However, an exaggerated inflammatory response mediated by alveolar macrophages, pulmonary vascular endothelium and neutrophils has been shown to play a critical role (Kooguchi, Hashimoto et al. 1998; Chignard and Balloy 2000; Finigan 2009).

1.2. Health and economic impact of acute lung inflammation

Acute lung inflammation is associated with a number of infectious and surgical conditions and results in very high mortality. The incidence of acute lung injury in has been estimated to be approximately 17-64 per 100,000 person per year (MacCallum and Evans 2005). In addition to high mortality of up to 40%, acute lung injury can also lead to neuromuscular, cognitive, and psychological dysfunction (Rubenfeld and Herridge 2007). Lung inflammation is associated with several bacterial and viral diseases in animals with high incidence and mortality (Ribble, Meek et al. 1995). The economic burden of treatment and preventive measures of lung inflammation in cattle is reported to be approximately \$1 billion every year in the United States alone (Ribble, Meek et al. 1995). Therefore, there is a need to develop novel therapeutic strategies for the treatment of acute lung injury.

1.3. General mechanism of acute lung inflammation

Acute lung inflammation is caused by both local and systemic insults (Downey, Dong et al. 1999). Although alveolar macrophages play an important role in initiation and regulation of inflammatory response, neutrophils are central to inflammation-induced tissue injury.

Neutrophils are recruited in response to inflammatory stimuli or chemotactic molecules released by alveolar macrophages through a complex and sequential interaction with endothelial cells (Stephens, Milne et al. 2008). Endothelial cells, in addition to facilitating neutrophil recruitment to sites of inflammation, secrete cytokines and reactive oxygen species which potentiate the inflammatory response (Bhatia and Moolchhala 2004). At the site of inflammation, neutrophils release proteases and reactive oxygen species which are considered as the main causes of significant tissue damage associated with many inflammatory diseases and acute lung injury (Grommes and Soehnlein 2011). Neutrophils recruited to the site of inflammation combat microbes and produce several cytokines to potentiate the inflammatory response (Cassatella 1999).

1.4. Neutrophils - biology, recruitment and function

Neutrophils are the most abundant leukocytes and an important cell in the host innate immune system. These are the first cells to be recruited to the sites of inflammation and their main function is to phagocytose and kill pathogens. Neutrophils differentiate from pluripotent haematopoietic stem cells, and fully differentiated neutrophils show a characteristic multilobed nucleus and have a short life span (Opferman 2007). The half life of neutrophils in circulation is 6-8 h (Fliedner, Cronkite et al. 1964). If the neutrophils are not activated and recruited to the sites of inflammation, they return to liver, spleen and bone marrow where they undergo spontaneous apoptosis and are removed by tissue macrophages through phagocytosis (Martin,

Burdon et al. 2003). In an adult human being, the daily turnover of neutrophils is reported to be 10^{11} (Athens, Haab et al. 1961).

1.4.1. Recruitment of neutrophils

Interaction between endothelial cells and neutrophils is a critical event in the recruitment of neutrophils to sites of inflammation. The neutrophil-endothelial interaction is mediated by adhesion molecules such as selectins, integrins, intercellular adhesion molecules (ICAMs), vascular cell adhesion molecules (VCAMs), and platelet-endothelial cell adhesion molecule-1 (PECAM-1). The first event in the interaction is rolling or tethering of neutrophils on the endothelium which is initiated through increased expression of selectins by activated endothelial cells (Kansas 1996). This is followed by conformational changes in neutrophil integrins that lead to a second event called firm adhesion or arrest (Salas, Shimaoka et al. 2004). Specifically, $\beta 2$ integrins play a critical role in neutrophil adhesion and the ligand for $\beta 2$ integrins on endothelial cells is intercellular adhesion molecule-1 (ICAM-1) (Luscinskas and Lawler 1994; Malik and Lo 1996). In lungs, both $\beta 2$ integrin dependant and independent pathway mediated by $\beta 1$ integrin are activated depending on the stimulus. In lipopolysaccharide-induced acute lung injury, $\beta 2$ integrin and ICAM-1 interaction mediates neutrophil transmigration (Doerschuk, Tasaka et al. 2000; Burns, Smith et al. 2003). Adhesion to endothelial cells is followed by extravasation of neutrophils from a blood vessels which is mainly mediated by PECAM-1 (Newman 1997).

1.4.2. Apoptosis

Apoptosis or programmed cell death is required for normal development of multicellular organisms and maintenance of homeostasis. Impairment in the execution of apoptosis leads to developmental and immunological disorders, neurodegeneration, and cancer (Fuchs and Steller 2011). Another form of cell death called ‘necrosis’ is characterized by cellular swelling,

compromised cell membrane integrity, lysis of cells resulting in release of intracellular contents. In contrast, apoptosis is characterized by cell shrinkage, condensation of chromatin, and intact cell membrane (Majno and Joris 1995; Saraste and Pulkki 2000). Extrinsic pathway of apoptosis induced by extracellular factors is mediated by cell surface receptors belonging to tumor necrosis family receptor family (Pan, O'Rourke et al. 1997). The intrinsic pathway induced by radiation and cellular stresses is mediated mainly by mitochondria. However, in both the pathways caspase activation plays an important role in execution of apoptosis (Elmore 2007).

1.4.2.1. Neutrophil apoptosis

Apoptosis is required for maintaining homeostasis by removal of aged and/or abnormal cells (Bianchi, Dockrell et al. 2006). Neutrophils are terminally differentiated cells and in the absence of any activation they undergo spontaneous apoptosis (Savill 1997). The process of spontaneous apoptosis is important to maintain the normal neutrophil count in circulation (Dancey, Deubelbeiss et al. 1976; Walker and Willemze 1980; Shi, Gilbert et al. 2001). This is because of differential expression of pro- and anti-apoptotic proteins in unactivated neutrophils. The short life span of neutrophils is due to higher levels of proapoptotic Bcl-2 family proteins such as Bax and Bak (Weinmann, Gaehtgens et al. 1999; Moulding, Akgul et al. 2001). In addition, unstimulated neutrophils don't express or express very low levels of antiapoptotic proteins and these have relatively short half lives (Moulding, Akgul et al. 2001; Gardai, Hildeman et al. 2004). In addition to maintaining normal neutrophil count in circulation, the neutrophil apoptosis is also critical for safe disposal of activated neutrophils from the site of inflammation without the release of harmful contents that minimizes tissue injury (Savill and Fadok 2000). Extravasated neutrophils are also recirculated back to lymph nodes (Schwab, Chiang et al. 2007).

1.4.2.2. Mechanism of neutrophil apoptosis:

Both extrinsic and intrinsic pathways are involved in regulating neutrophil apoptosis. The extrinsic pathway of apoptosis is induced in response to ligand binding to cell surface death receptors of the tumor necrosis factor (TNF) receptor superfamily or Fas receptors (Ashkenazi and Dixit 1998). The constitutive spontaneous apoptosis of neutrophils is reported to be mediated through interaction of Fas ligand with its receptor (Liles, Kiener et al. 1996; Renshaw, Timmons et al. 2000). However, there are contrasting reports about the role of Fas receptors. Although neutrophils have been shown to express functional Fas receptors (CD95, APO-1), the receptors are not involved in spontaneous neutrophil apoptosis (Liles, Kiener et al. 1996; Brown and Savill 1999). Moreover, spontaneous neutrophil apoptosis was unaffected in mice deficient in Fas receptor or Fas ligand (Fecho and Cohen 1998). Neutrophils also express TNF-related apoptosis-inducing ligand (TRAIL) receptors and undergo apoptosis when treated with their ligands (Renshaw, Parmar et al. 2003).

The intrinsic pathway is mediated through mitochondria and activated in response to ultraviolet radiation, cytotoxic agents, and damage by reactive oxygen species (Susin, Daugas et al. 2000). Although neutrophils have very few mitochondria (Bainton, Ulliyot et al. 1971; Clark, Vaughan et al. 1980), the mitochondrial or intrinsic pathway also plays an important role in neutrophil apoptosis. This has been shown by loss of mitochondrial transmembrane potential during the early stages of neutrophil apoptosis (Martin, Dransfield et al. 2001; Fossati, Moulding et al. 2003). In addition low levels of cytochrome-*c* are sufficient for caspase activation in the mitochondrial-dependant pathway of neutrophil apoptosis (Murphy, O'Neill et al. 2003).

Caspases mediate both extrinsic and intrinsic pathways of neutrophil apoptosis (Simon 2003). Neutrophils express regulatory caspases such as caspase-4, -8 and effector caspases such

as caspases-3 (Yamashita, Takahashi et al. 1999; Santos-Beneit and Mollinedo 2000). Caspase-3 is an effector caspase that plays an important role in neutrophil apoptosis, and this has been further confirmed by impaired apoptosis in neutrophils isolated from caspase-3 deficient mice (Woo, Hakem et al. 1998; Daigle and Simon 2001).

1.4.2.3. Changes in apoptotic neutrophils

Neutrophils undergoing apoptosis show a number of changes that lead to the loss of functional properties. Several cell surface receptors that are important for neutrophil functions are down regulated during the process of apoptosis (Dransfield, Buckle et al. 1994). (Homburg, de Haas et al. 1995). Plasma membrane symmetry is altered resulting in the translocation of phosphatidylserine from the cytoplasmic side to extracellular surface (Naito, Nagashima et al. 1997). Apoptotic neutrophils lose their functional properties because of inactivation of many intracellular pathways and loss of cell surface receptors (Akgul, Moulding et al. 2001). Degranulation ability of neutrophils is impaired or suppressed during the process of neutrophil apoptosis (Whyte, Meagher et al. 1993). A number of pro-inflammatory functions such as chemotaxis, secretion of reactive oxygen species and cytokines, and ability to phagocytose are suppressed in apoptotic neutrophils (Whyte, Meagher et al. 1993; Kobayashi, Voyich et al. 2003). The suppression of proinflammatory functions of neutrophils during the process of apoptosis is vital in minimizing tissue damage (Whyte, Meagher et al. 1993). In addition, during the process of apoptosis the plasma membrane of neutrophils remains intact, which prevents the release of harmful contents (Savill, Wyllie et al. 1989). The suppression of proinflammatory functions is important not only to prevent tissue injury, but also for initiation of resolution (Kobayashi, Voyich et al. 2005).

1.5. Role of neutrophils in acute lung inflammation

Although neutrophils are an important component of the host defence system, activated neutrophils contribute to significant tissue damage. Neutrophil infiltration is a characteristic feature in lung inflammatory diseases such as acute lung injury, chronic obstructive pulmonary disease, bronchiectasis, bronchiolitis, and cystic fibrosis (Cowburn, Condcliffe et al. 2008). The role of neutrophils in acute lung injury has been well established by several studies in animal models and epidemiological studies. Accumulation of activated neutrophils in inflamed lung is considered as the hallmark of acute lung injury (Downey, Dong et al. 1999). Neutrophils are recruited from the circulation in response to inflammatory stimuli through a cascade of interactions with vascular endothelial cells. They release antimicrobial molecules such as reactive oxygen species and proteolytic enzymes which have been implicated in neutrophil-induced tissue injury (Moraes, Zurawska et al. 2006). Neutrophils have been found to be predominant cell types in bronchoalveolar lavage fluid from patients diagnosed with acute lung injury or acute respiratory distress syndrome (Steinberg, Milberg et al. 1994). An increased neutrophil count in bronchoalveolar lavage fluid has also been associated with poor prognosis in human patients with acute respiratory distress syndrome further corroborating the role of neutrophils in acute lung injury (Steinberg, Milberg et al. 1994). In addition, in animal models severity of acute lung injury decreases following the depletion of neutrophils (Folz, Abushamaa et al. 1999; Abraham, Carmody et al. 2000). Depletion of neutrophils following induction of sepsis also resulted in reduced bacteremia and a marked reduction in serum levels of proinflammatory cytokines (Hoesel, Neff et al. 2005). Furthermore, occurrence of acute lung injury in neutropenic individuals has been reported to be infrequent (Laufe, Simon et al. 1986).

1.5.1. Neutrophil granule contents in pathogenesis of lung inflammation

Neutrophils contain four granules, namely primary (azurophilic), secondary (specific), tertiary (gelatinase) granules, and secretory vesicles. The primary granules contain mediators such as proteolytic enzymes, myeloperoxidase, cathepsin G and defensins. The secondary granules act as storage site for lactoferrin, and tertiary granules contain matrix metalloproteinases (MMPs). The secretory vesicles contain human serum albumin (Faurschou and Borregaard 2003; Lacy 2006). Activated neutrophils release a number of potentially harmful substances such as proteolytic enzymes and reactive oxygen and nitrogen species (Moraes, Zurawska et al. 2006). The role of proteolytic enzymes such as elastase and metalloproteinases in the pathogenesis of acute lung injury has been extensively studied. Elastase is released by activated neutrophils and it causes apoptosis of alveolar epithelial cells by binding to proteinase-activated receptors (Suzuki, Moraes et al. 2005). Levels of neutrophil elastase are elevated even in plasma of patients with acute lung injury or acute respiratory distress syndrome. Moreover, there is a correlation with increased levels of plasma elastase and degree of lung injury (Donnelly, MacGregor et al. 1995). Inhibition of neutrophil elastase is beneficial in amelioration of acute lung injury (Inoue, Omodani et al. 2009). Proteases also cause significant damage to extracellular matrix of the lung (Pittet, Mackersie et al. 1997). Matrix metalloproteinases (MMPs) secreted by neutrophils also play an important role in the pathogenesis of acute lung injury. Increased levels of MMPs have been reported both from bronchoalveolar lavage fluid (Torii, Iida et al. 1997) and plasma (Steinberg, Fink et al. 2001) of patients with acute lung injury, and there was a correlation between levels of MMPs and severity of the disease (Fligiel, Standiford et al. 2006). Elevated levels of matrix metalloproteinases have been found in epithelial lining fluid of patients with acute respiratory distress syndrome ((Delclaux, d'Ortho et al. 1997). Furthermore,

lung injury was ameliorated following the inhibition of elastase and metalloproteinases after cardiopulmonary bypass in pigs (Carney, Lutz et al. 1999). Neutrophil granule contents play an important role in lung injury following administration of *Streptococcus pyogenes*, and depletion of neutrophils resulted in amelioration of lung damage (Soehnlein, Oehmcke et al. 2008).

1.5.2. Neutrophil apoptosis in acute lung inflammation

A balance between neutrophil survival and apoptosis at sites of inflammation is important for the successful resolution of inflammation and to minimize concurrent tissue damage. This balance is impaired in many inflammatory diseases (Leitch, Duffin et al. 2008). Neutrophil apoptosis influences dynamics of organ inflammation. The resolution of inflammation appears to be linked to the clearance of the apoptotic neutrophils by macrophages (Savill, Henson et al. 1989). Therefore, targeting of apoptotic pathway in neutrophils by pharmacological agents and administration of apoptotic cells in sepsis have been shown to improve survival in animal models (Rossi, Sawatzky et al. 2006; Ren, Xie et al. 2008). Although neutrophils are terminally differentiated cells and programmed to undergo constitutive apoptosis, apoptosis in activated neutrophils is delayed at the site of inflammation, a delay which aggravates acute lung injury (Matute-Bello, Liles et al. 1997). The delayed apoptosis is due to the effects of pathogen-derived products and inflammatory cytokines on neutrophils at the site of inflammation (Colotta, Re et al. 1992). The spontaneous apoptosis was significantly reduced in neutrophils from bronchoalveolar lavage fluid of ARDS patients. In addition, bronchoalveolar lavage fluid from ARDS patients delayed spontaneous apoptosis of normal human neutrophils (Matute-Bello, Liles et al. 1997; Lesur, Kokis et al. 2000). It has been shown that in community-acquired pneumonia there is decreased spontaneous apoptosis accompanied by increased activation of neutrophils (Droemann, Aries et al. 2000). In the sputum of patients with chronic obstructive pulmonary

disease, spontaneous apoptosis of neutrophils is reduced along with activation of NF- κ B (Brown, Elborn et al. 2009). Resolution of lung inflammation in respiratory distress syndrome in newborn infants was associated with higher rates of apoptosis and proapoptotic bronchoalveolar lavage fluid (Kotecha, Mildner et al. 2003). In animal models of acute lung injury, neutrophil-mediated acute lung injury can be ameliorated by induction of neutrophil apoptosis (Sookhai, Wang et al. 2002). In addition to minimizing tissue injury, apoptotic neutrophils also trigger macrophages to switch into an anti-inflammatory and pro-resolution phenotype (Fox, Leitch et al. 2010). Therefore, neutrophil apoptosis is intricately linked to the outcomes of inflammation in an organ.

1.6. Modulation of neutrophil apoptosis

1.6.1. Host-derived inflammatory mediators

NF- κ B regulates transcription of a number of pro-inflammatory genes as well as neutrophil apoptosis. Activation of NF- κ B delays spontaneous apoptosis of neutrophils (Ward, Chilvers et al. 1999). The inhibitory effects on spontaneous apoptosis are due to NF- κ B mediated transcription of survival factors in activated cells and inhibitors of NF- κ B have been shown to abolish this effect (Hallett, Leitch et al. 2008).

A number of factors derived from both host cells and pathogens at the sites of inflammation regulate survival and apoptosis of neutrophils (Colotta, Re et al. 1992; Lee, Whyte et al. 1993). The host cell-derived factors include proinflammatory cytokines such as granulocyte-macrophage colony-stimulating factor, granulocyte colony stimulating factor, TNF- α , interleukin-1 β (IL-1 β), and (IL-6) which are released by activated immune cells at the site of inflammation (Colotta, Re et al. 1992). The inhibitory effects of these mediators on neutrophil apoptosis happen through pathways such as phosphatidylinositol 3-kinase (PI3-K), mitogen

activated protein kinase/extracellular signal regulated kinases (MAPK/ERK), and p38 mitogen activated protein kinase (p38MAPK). GM-CSF delays neutrophil apoptosis through phosphorylation of intracellular tyrosine kinases such as Lyn and Jak2 (Brizzi, Aronica et al. 1996; Wei, Liu et al. 1996; Al-Shami, Mahanna et al. 1998). Downstream pathways activated by tyrosine kinase phosphorylation include PI3-K and MAPK (Vlahos, Matter et al. 1995; Klein, Rane et al. 2000; Simon 2003). Activation of MAPK/ERK by GM-CSF leads to delay in spontaneous apoptosis of neutrophils (Nolan, Duffy et al. 1999). PI3-K activation leads to increased levels of Mcl-1 that delays spontaneous apoptosis (Moulding, Akgul et al. 2001). Data from animal models show that G-CSF delays neutrophil apoptosis through activation of PI3-K and MAPK/ERK pathways (Klein, Rane et al. 2000). In addition to GM-CSF, TNF- α also induces tyrosine phosphorylation of MAPK (Waterman and Sha'afi 1995; Nahas, Molski et al. 1996). However, unlike other inflammatory mediators TNF- α is a death receptor ligand, which has both pro-apoptotic and anti-apoptotic effects. Short-term exposure to TNF- α induces apoptosis whereas long time exposure results in delayed apoptosis. Delayed apoptosis by TNF- α is due to activation of anti-apoptotic signal through NF- κ B activation (Murray, Barbara et al. 1997). Most of the proinflammatory cytokines-induce delay in spontaneous apoptosis of activated neutrophils through upregulation of anti-apoptotic Bcl-2 proteins such as A1, Mcl-1, and Bcl-xL (Hamasaki, Sendo et al. 1998; Dzhagalov, Dunkle et al. 2008). Activation of MAPK/ERK and phosphoinositide-3-kinase (PI3K) pathways also inhibit the mitochondrial pathway of apoptosis (Luo and Loison 2008). Expression of Mcl-1 has been reported to correlate with survival of human neutrophils (Moulding, Quayle et al. 1998). There are ambiguous reports about the role of p38 MAPK in neutrophil apoptosis. Activation of p38 MAPK results in either accelerated or delayed apoptosis in neutrophils (Khreiss, Jozsef et al. 2002; Alvarado-

Kristensson, Melander et al. 2004). In addition to the above mentioned pathways, there are roles for protein kinase-A (PKA), protein kinase-B (PKB) and protein kinase-C (PKC) in the regulation of neutrophil apoptosis. Activation of PKA and PKB lead to increased levels of anti-apoptotic messenger molecules such as cyclic adenosine monophosphate (cAMP) (Rossi, Cousin et al. 1995). cAMP delays spontaneous apoptosis of neutrophils and this effect is abolished by inhibition of protein kinase A (Parvathenani, Buescher et al. 1998). However, protein kinase C exerts both pro and anti-apoptotic effects on neutrophils (Webb, Wang et al. 2000). In contrast to inflammatory cytokines, reactive oxygen species promote neutrophil apoptosis since the neutrophils from patients with a genetic defect in NADPH oxidase show delayed spontaneous apoptosis (Kasahara, Iwai et al. 1997). Collectively, the data show a complex regulatory profile for various inflammatory mediators in the regulation of apoptosis in neutrophils.

1.6.2. Pathogens and pathogen derived factors

In addition to host-derived inflammatory mediators, a number of microbial products such as lipopolysaccharide and other toxins also delay spontaneous apoptosis of neutrophils (DeLeo 2004). The toll-like receptor ligands such as lipopolysaccharide and lipotechoic acid delay spontaneous apoptosis of neutrophils (Sabroe, Prince et al. 2003; Lotz, Aga et al. 2004). Lipopolysaccharide leads to activation of NF- κ B and subsequent transcription of anti-apoptotic factors (Castro-Alcaraz, Miskolci et al. 2002). Enterotoxins of staphylococcus, verotoxin derived from *E. coli*, water soluble proteins from *Helicobacter pylori* also suppress neutrophil apoptosis (Liu, Akahoshi et al. 1999; Moulding, Walter et al. 1999; Kim, Kim et al. 2001). Various pathogens themselves have direct effect on the neutrophil apoptosis. *Anaplasma phagocytophilum* and *Chlamydia pneumoniae* not only survive and replicate within the neutrophils but they also delay the spontaneous apoptosis of neutrophils (Yoshiie, Kim et al.

2000; van Zandbergen, Gieffers et al. 2004). Pathogens such as *Streptococcus pyogenes* modulate the process of neutrophil apoptosis resulting in lysis of cells leading to significant tissue damage (Kobayashi, Braughton et al. 2003). In contrast to the above reports, bacterial species such as *Mycobacterium tuberculosis* promote human neutrophil apoptosis (Perskvist, Long et al. 2002).

1.7. Role of endothelium in acute lung inflammation

The vascular endothelium consists of a single continuous layer of simple squamous epithelial cells (ECs), and it is a highly specialized metabolically active organ possessing numerous physiological, immunological, and synthetic functions (Orfanos, Mavrommati et al. 2004). Endothelial cells are activated by various inflammatory cytokines and produce cytokines. Proinflammatory cytokines such as TNF- α and IL-1 activate signaling pathways in endothelial cells that promote thrombosis and inflammation (Mantovani, Bussolino et al. 1997). TNF- α induces production of neutrophil chemoattractants such as IL-8 in pulmonary endothelial cells through activation of p38 mitogen-activated protein kinase pathway (Hashimoto, Gon et al. 2001). Interaction between cytokines and endothelial cells plays an important role in pathogenesis of acute lung injury. Pulmonary vascular endothelial cells isolated from ARDS patients show increased expression of TNF-R2 receptors and a higher production of IL-6 and IL-8 than control, which indicates their activation (Grau, Mili et al. 1996). Accumulations of activated neutrophils and pulmonary edema due to increased microvascular permeability have been considered as the hall mark of acute lung injury (Ware 2006). Increased microvascular permeability occurs due to endothelial injury and formation of intercellular gaps between endothelial cells (Hurley 1982). Increased vascular permeability has been directly correlated with severity of acute lung injury and neutrophil counts in bronchoalveolar lavage fluid (Murray,

Matthay et al. 1988; Sinclair, Braude et al. 1994). In addition to other cell types, pulmonary vascular endothelial cells also produce potentially harmful substances such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Bhatia and Moochhala 2004). Many endothelial cells including those in the lung contain cytoplasmic structures called Weibel-Palade bodies. The Weibel-Palade bodies contain preformed IL-8, P-selectin and von Willebrand factor, and are released upon stimulation with cytokines and lipopolysaccharide. The release and engagement of these inflammatory mediators is critical for the very early inflammatory events in the blood vessels. Therefore, endothelial cells are one of the critical regulators of acute inflammation through recruitment of neutrophils and maintenance of vascular barrier.

1.8. Contribution of alveolar macrophages in acute lung inflammation

Alveolar macrophages (AM) are the resident immune cells of the respiratory system acting as one of the first lines of defence against infectious, toxic, or allergic molecules invading the respiratory system. Alveolar macrophages play central role in initiation and regulation of acute lung injury through the release of several inflammatory mediators such as cytokines, chemokines and arachidonate metabolites (Rubins 2003). Activated alveolar macrophages also contribute to damage to alveolar epithelium resulting in alveolar barrier dysfunction (Frank, Wray et al. 2006). In a mouse model of bacterial pneumonia, depletion of alveolar macrophages decreased neutrophil recruitment, chemokine release, and lung injury during the early inflammatory response. However, there was aggravated lung injury at 48 h after depletion of alveolar macrophages because of impaired clearance of both bacteria and neutrophils from the site of inflammation. Mice upon depletion of alveolar macrophages showed low mortality within 24 h of infection but high mortality at a later time, in contrast to non-AM depleted mice (Kooguchi, Hashimoto et al. 1998). However, in non-inflammatory lung injury caused by the

selective ablation of alveolar epithelial type II cells, alveolar macrophages have been shown to play a protective role ((Miyake, Kaise et al. 2007). They also play an important role in resolution of the inflammatory process (Haslett 1999). The alveolar macrophages aid in resolution of lung inflammation through phagocytosis of apoptotic neutrophils, which minimizes tissue damage. Phagocytosis of apoptotic neutrophils promotes production of anti-inflammatory cytokines to initiate resolution of inflammation (Haslett 1999). Taken together, alveolar macrophages have a complex role in acute lung injury.

1.9. Retinoids

The term ‘retinoids’ was coined by Michael Sporn and his colleagues in 1976 (Sporn, Dunlop et al. 1976). It refers to natural vitamin A and the compounds that are structurally related to vitamin A. These include preformed vitamin A alcohol, retinol and its aldehyde, retinal, its acid *trans* retinoic acid or tretinoin, and provitamin β -carotene. Vitamin A is an essential nutrient that cannot be synthesised in the body and hence it must be provided through diet (Blomhoff and Blomhoff 2006). Retinyl esters in animal products and β -carotene in plants are the two types of precursors of vitamin A (Davidovici, Tuzun et al. 2007). Retinoids are required for several physiological functions such as vision, immune function, reproduction, maintenance of epithelial integrity, and differentiation (De Luca 1991). Deficiency of vitamin A leads to various disorders such as night blindness, fetal reabsorption, skeletal disorders, atrophy of testes, and immunodeficiency that leads to increased morbidity and mortality (Shils, 2006).

1.9.1. Absorption and metabolism of retinoids

Dietary retinyl esters and β -carotene undergo metabolism within the intestine, both extracellularly in the lumen and intracellularly in intestinal epithelial cells (Blaner and Olson, 1994). The dietary β -carotene is converted to retinal by the enzyme 15-15¹ β -carotene oxygenase,

which is present in the intestinal epithelium followed by reduction to retinol (Olson and Hayaishi 1965; Li and Tso 2003). The retinol packed in chylomicrons is released into the lymphatic system and taken up by the liver (Blomhoff, Helgerud et al. 1982). The retinol is stored as retinyl esters in liver and released into circulation for delivery to other tissues. In the circulation, retinol is bound to retinol-binding protein (RBP). It is absorbed through passive diffusion and bound to cellular retinol binding protein II (CRBP II) (Fields, Soprano et al. 2007). Retinol is metabolised intra-cellularly forming two active metabolites: all-*trans* retinoic acid and 9-*cis* retinoic acid. The active metabolites bind to retinoid receptors to exert their effects (Niederreither, Fraulob et al. 2002; Lin, Zhang et al. 2003). Retinol is also converted to retro-retinoids for which receptors have not been identified yet (Buck, Derguini et al. 1991; Geissmann, Revy et al. 2003).

1.10. Mechanism of action

1.10.1. Retinoid receptors

Retinoids exert their effects by binding to two major families of nuclear receptors superfamily, retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Both RARs and RXRs are comprised of three subtypes, namely α , β , and γ and several isoforms have been identified within each subtype. Subtypes of RARs differ in their amino terminal end and each subtype is coded by a different gene (Wei 2003). The relative levels of these subtypes vary between different tissues. All-*trans* retinoic acid (ATRA) binds to RARs whereas 9 *cis*-retinoic acid (9-CRA) binds to both RARs and RXRs (Kuenzli, Tran et al. 2004).

1.10.2. Structure of retinoid receptors

RARs have a structure that is common to other nuclear receptors. They have an amino terminal activation domain called AF-1, a central DNA binding domain and a carboxy terminal ligand binding domain (LBD). The ligand binding domain also contains an additional activation

domain called AF-2 (Tenbaum and Baniahmad 1997). The DNA binding domain is conserved among the three subtypes of each family and it determines the specificity of DNA response element recognition and binding affinity to respective DNA response elements (Chambon 1996). LBD consists of 12 helices and short β turn (de Lera, Bourguet et al. 2007). Retinoid receptors function as transcription factors after binding to their respective ligands and regulate the gene expression. RARs form heterodimers with RXRs, whereas RXRs form either homodimers or heterodimers with other nuclear receptors (Mangelsdorf and Evans 1995; Moras and Gronemeyer 1998).

1.10.3. Intracellular signaling

Retinoid receptors function as ligand activated transcription factors. In the unligated state, RARs are bound to a group of proteins called corepressors. The members of this group of proteins include the nuclear receptor corepressor (NCoR) and silencing mediator for retinoid and thyroid hormone receptor (SMRT) (Hu and Lazar 1999; Nagy, Kao et al. 1999). Binding of corepressors leads to recruitment of histone deacetylases leading to local histone deacetylation, restricted access to transcription factors and reduced gene expression (Heinzel, Lavinsky et al. 1997; Kouzarides 2002).

A conformational change is induced in the helix 12 of AF-2 domain of RARs after the ligand binding (Renaud, Rochel et al. 1995). This leads to recruitment of a group of nuclear proteins called coactivators (Moras and Gronemeyer 1998). The p160 family is a large coactivator family, which interacts with RARs. This family of co-activators comprises of 3 proteins namely, steroid receptor coactivators-1 (SRC-1), SRC-2, and SRC-3. Another family of coactivators is referred to as cointegrators of which E1A-associated 300kDa protein is an example (Leo and Chen 2000). In addition to these two, another unique coactivator, cellular

retinoic acid binding protein II (CRAB II) has been identified (Delva, Bastie et al. 1999). These co-activators either possess an intrinsic histone acetylase activity (HAT) or are associated with histone acetylases (HATs) (Yuan, Condorelli et al. 1996; Spencer, Jenster et al. 1997).

Acetylation of N-terminal tails of histones by these HATs leads to neutralisation of charge of histones, loosening the binding of histone proteins to negatively charged DNA and nucleosome to nucleosome interaction, relaxing the chromatin structure (Bannister and Kouzarides 1996). This is followed by the recruitment of general transcription factors, TFIID complexes and RNA poly II to form transcription pre-initiation complex (PIC). Completion of formation of pre-initiation complex initiates the transcription of target genes (Chen and Privalsky 1997) .

Retinoid-mediated signal transduction is down regulated by degradation of RARs and RXRs through the ubiquitin-proteasome system. Retinoid receptors are ubiquitinated at multiple sites and directed for proteasomal degradation. Phosphorylation also regulates the retinoid signalling. Phosphorylation leads to recruitment of ubiquitin ligases and subsequent degradation (McGrane 2007).

There have been few studies to determine functions of RAR subtypes using knock-out mice. These studies have reported that RAR single subtype knockout animals are not only viable but they also show some features of postnatal and fetal vitamin A deficiency syndromes. For example, RAR α -knockout males show degeneration of the seminiferous tubule epithelium resulting in inhibition of spermatogenesis (Li *et al.*, 1993; Lufkin *et al.*, 1993). RAR β -knockout mice show abnormalities of vitreous body in eyes (Grondona, Kastner et al. 1996), locomotion and motor incoordination (Krezel, Ghyselinck et al. 1998). RAR γ -null mice show skeletal and epithelial defects (Lohnes, Kastner et al. 1993; Ghyselinck, Dupe et al. 1997; Chapellier, Mark et al. 2002). However, animals with knock out of more than one receptor subtype have been found

to be less viable and show most of the manifestations of the vitamin A deficiency (Lohnes, Mark et al. 1994; Lohnes, Mark et al. 1995; Luo, Sucov et al. 1996). The data show that loss of retinoid receptors has serious physiological implications for the host.

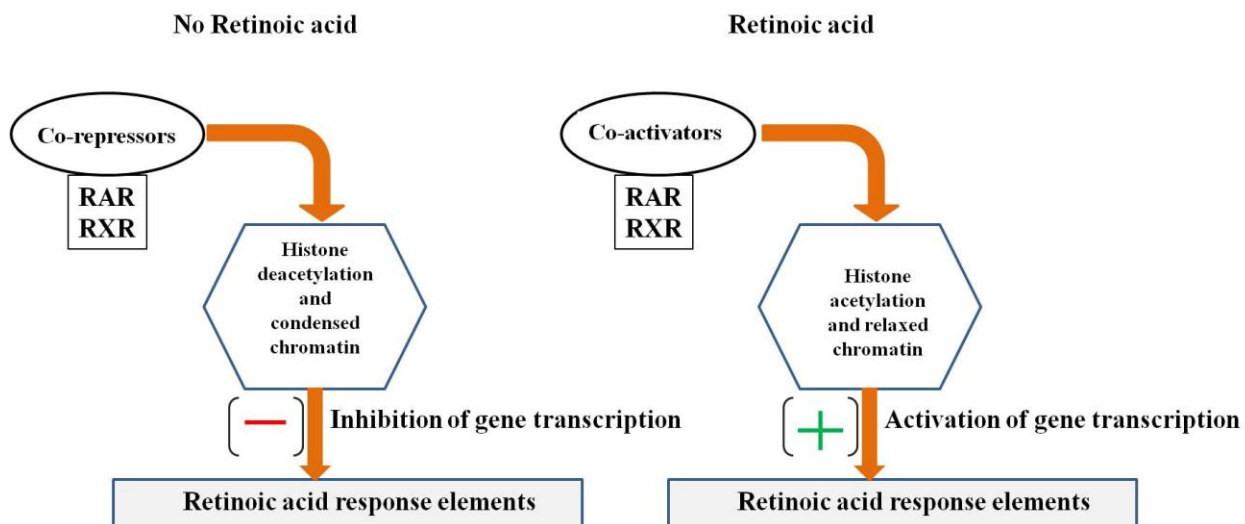


Figure 1.1. Mechanism of action of retinoic acid. In unliganded state, RARs are bound to repressors, leading to histone deacetylation, condensed chromatin leading to inhibition of gene transcription. Upon ligand binding RARs bind to co-activators leading to histone acetylation, relaxation of chromatin and activation of gene transcription.

1.11. Expression of retinoid receptors

The expression levels of retinoid receptors vary depending on the developmental stage, tissue, and the cell type, which possibly indicates distinct function for each receptor subtype. RAR α expression has been reported in almost all the tissues, where as RAR β and RAR γ are predominantly expressed in neural tissues and skin, respectively (Mangelsdorf 1994; Chambon 1996). Analysis of the expression pattern of RAR genes in fetal and newborn rat lung showed expression of RAR α , RAR β , and RAR γ with a significant increase in the mRNA levels of both RAR α and RAR β in fetal lungs indicating their role in lung development (Grummer, Thet et al.

1994). A study on the temporal and spatial expression pattern of retinoid receptors in mice lungs collected at different post natal stages reported the expression of RAR α 1, β 2, β 4, and γ 2 in postnatal mouse lung. These RAR isoforms were localized to bronchial epithelium, bronchial and vascular smooth muscle, pleura, and scattered cells within the alveolar regions, some of which were found to have the characteristic morphology of type II pneumocytes. All identified RAR isoforms were upregulated in the neonatal period compared to their expression in the adult suggesting their role in alveologenesis (Hind, Corcoran et al. 2002). Both RAR α and RAR β subtypes are abundantly expressed in lungs of rats (Rees, Daly et al. 1989). All six retinoid receptor genes are widely expressed in lymph nodes of children, and the levels of mRNA were increased with the development of children (Wei, Yang et al. 2007). In the same study it was shown that in *in vitro* culture, all-*trans* retinoic acid promotes the maturation and activation of the B cells. This explains the possible role of retinoid receptors in antibody enhancing effect of vitamin A. *There are no data on the expression pattern of retinoid receptors in any of the domestic animal species.*

1.11.1. Altered expression of retinoid receptors

Expression of retinoid receptor is altered in various pathophysiological conditions. It has been shown that little or no RAR α protein was present in resting polymorphonuclear leukocytes (PMNs). However, the constitutive mRNA encoding RAR α in these cells is rapidly translated to protein upon activation by platelet activating factor (PAF) (Yost, Denis et al. 2004). In addition to PAF, TNF- α , lipopolysaccharide and fMLP also induced the synthesis of RAR α protein. The synthesis of RAR α protein has been found to be regulated at the translational level by mTOR, as rapamycin, a blocker of mTOR, was found to inhibit the synthesis of RAR α protein in PAF stimulated PMNs.

Expression of retinoic acid receptors is altered in various pathological conditions. The epithelial expression of RAR γ , RXR α and RXR γ has been shown to be greater in chronic inflammatory conditions of lung such as asthma as compared to healthy individuals (Druilhe, Zahm et al. 2008). Furthermore, retinoid receptor expression also correlated positively with the proportion of morphologically intact epithelium. Vitamin A status also alters the expression levels of retinoic acid receptors. The expression levels of RAR β , and RAR γ were significantly lowered whereas levels of RAR α were upregulated in vitamin A deficient animals. When vitamin A was supplemented to deficient animals, the expression was restored to near normal levels (Verma, Shoemaker et al. 1992). In addition to changes in their expression, retinoid receptor subtypes are redistributed within the cells following the cellular activation. RXR α translocated from nucleus to cytoplasm accompanied by a reduction in nuclear RXR α protein levels in hepatic tissue of endotoxin-treated mice. Expression of six hepatic genes regulated by RXR α were markedly suppressed after LPS treatment (Ghose, Zimmerman et al. 2004). In a comprehensive analysis of retinoic acid receptors and retinoid X receptors in samples of non-small cell lung cancer decreased levels of all three subtypes of both RARs and RXRs in neoplastic cells has been reported (Brabender, Metzger et al. 2005). Expression of RAR α mRNA is upregulated in canine mast cell tumor cell lines and treatment with ATRA caused significant growth inhibition in these cell lines (Miyajima, Watanabe et al. 2006). In a mouse acute phase response model, expression of all three subtypes of retinoid X receptors decreases in heart (Feingold, Kim et al. 2004). *However, currently no data are available on their expression in acute lung inflammation.*

1.12. Retinoids in inflammation and immunity

The critical role of vitamin A in the function of the immune system was identified in early 20th century. A number of studies in different animal models reported that vitamin A deficiency leads to impaired cell-mediated and humoral immune responses (Wiedermann, Hanson et al. 1993; Wiedermann, Hanson et al. 1993). Available literature also suggests that retinoids and their receptors have a profound influence on inflammatory response. They affect the skin barrier function, function of neutrophils, macrophages, natural killer cells, and T and B lymphocytes (Kuenzli, Tran et al. 2004).

In a rat model of colitis, vitamin A deficiency has been shown to amplify the inflammatory response through activation of NF- κ B and collagen formation. Inflammatory markers such as TNF- α and nitric oxide were higher in the vitamin A deficient group. The exaggerated inflammatory response was ameliorated by supplementation of vitamin A (Reifen, Nur et al. 2002). Vitamin A deficiency also resulted in increased number of white blood cells accompanied by enhanced granulocyte-mediated inflammation and increased the interferon- γ production. Furthermore, vitamin A deficient animals showed a stronger immediate reaction after subcutaneous antigen injection of histamine (Wiedermann, Chen et al. 1996). Macrophage-mediated inflammation was enhanced through increased production of IL-12 and IFN- γ in Vitamin A deficiency. However, it also impaired the ability of macrophages to ingest and kill bacteria (Stephensen 2001). The data show that deficiency of vitamin A either directly or through altered expression of retinoid receptors makes the host susceptible to exaggerated inflammatory responses.

1.13. Effects of retinoids on inflammatory cells

1.13.1. Effects on neutrophils

Vitamin A deficiency has been shown to affect the function of inflammatory cells. In vitamin A deficient animals neutrophils show impaired phagocytosis and killing of bacteria, decreased chemotaxis towards *P. aeruginosa* and fMLP, and reduced generation of active oxidative molecules. Neutrophil proteinase such as cathepsin G is decreased under vitamin A deficiency (Twining, Zhou et al. 1996). However, *in vitro* studies have reported that retinoids inhibit lysosomal enzyme release and production of superoxide production from human neutrophils (Camisa, Eisenstat et al. 1982). Incubation of human neutrophils with *all trans* retinoic acid inhibited production of reactive oxygen species and release of proteolytic enzymes from fMLP-activated human neutrophils ((Varani, Jones et al. 1991). Retinoids inhibited the respiratory burst and degranulation of stimulated human polymorphonuclear leucocytes, probably through the mediation of transforming growth factor β -induced differentiation of tracheal epithelial cells into squamous cells (Jetten, Shirley et al. 1986). Retinoic acid also affects the maturation and function of neutrophils. RAR α when unligated is a negative regulator of promyelocyte differentiation but promotes differentiation upon ligation (Kastner and Chan 2001). Down-regulation of RXR α is critical for the development of neutrophils from granulocyte/monocyte progenitors (Taschner, Koesters et al. 2007). Retinoids have also been shown to affect the expression of key adhesion molecules necessary for neutrophil chemotaxis. Adhesion molecule CD11b expression on circulating neutrophils decreased when patients with pustular psoriasis were treated with retinoid compounds (Sjogren, Stendahl et al. 2000).

1.13.2. Effects on monocytes and macrophages

All trans retinoic acid suppressed production of macrophage derived chemokine (MDC) and interferon-inducible protein 10 (IP-10)/CXCL10 in monocytes through c-Raf-MKK1/2-ERK/MAPK pathway (Tsai, Chang et al. 2008). *All trans* retinoic acid also significantly reduced the VLA-4-dependent migration of peripheral blood mononuclear cells activated in vivo in mercuric chloride-induced nephritis in Brown Norway rats. These effects could be due to modulation of cytoskeletal changes such as distribution of F-actin and VLA-4 integrin in focal contacts that are essential for cell adhesion. In addition, it also abrogates the redistribution of Rac1 and RhoA proteins which are implicated in cell motility (Escribese, Conde et al. 2008). Under normal physiological conditions RAR γ increases the production of inflammatory cytokines by macrophages. This was supported by impaired production of cytokines by macrophages isolated from RAR γ deficient mice in response to activation by toll-like receptor ligands (Dzhagalov, Chambon et al. 2007). Retinoic acid inhibits transcriptional activity of NF- κ B in monocytic cells stimulated by TNF- α and lipopolysaccharide (Chen, Ma et al. 2002).

1.13.3. Effects on other cells

Retinoic acid and other retinoid derivatives have also been shown to inhibit TNF- α induced production and enzymatic activity of matrix metalloproteinase-1 (MMP-1) and -13 in human chondrocytes (Ho, Lin et al. 2005). *All-trans* retinoic acid and its stereoisomer 9-cis retinoic acid inhibited the production of IL-4 induced eotaxin production by human bronchial epithelial cell (Takamura, Nasuhara et al. 2004). Treatment with the retinoic acid suppressed lipopolysaccharide-induced mRNA expression and protein levels of several pro-inflammatory cytokines by astrocytes (van Neerven, Nemes et al. 2010). Retinoic acid inhibits production of cytokines such as IFN- γ and TNF- α by lamina propria mononuclear cells (Bai, Lu et al. 2010). In

addition to the above mentioned effects, retinoids also affect the adaptive immune responses. Vitamin A enhances the survival and proliferation of both B and T cells (Ross and Gardner 1994).

1.13.4. Effects of retinoids on apoptosis

There have been many studies on the effects of retinoids on apoptosis of different cell types. Most of these studies have been done to determine the effects of retinoic acid on neoplastic cells and have shown pro-apoptotic effects. Retinoic acid also induces apoptosis in immune cells such as T lymphocytes through the stimulation of RAR γ . However specific stimulation of RAR α , has been shown to prevent apoptotic effect of retinoic acid (Szondy, Reichert et al. 1998). In contrast to pro-apoptotic effects on T lymphocytes, both *all-trans* retinoic acid and *9-cis*-retinoic acid have antiapoptotic effects on human eosinophils. The anti-apoptotic effect was attributed to downregulation of caspase 3 and inhibition of its enzymatic activity by retinoic acid (Ueki, Mahemuti et al. 2008). *However, there were no data on the effects of retinoic acid on neutrophil apoptosis.*

1.14. Retinoids and tissue inflammation

Vitamin A is needed to maintain the integrity of epithelium in various tissues (Gorodeski, Eckert et al. 1997; Filteau, Rollins et al. 2001). It is well known that breach of epithelial integrity can lead to excessive invasion by bacteria resulting in exacerbated inflammatory response (Kim 2011). Vitamin A deficiency can either exacerbate or inhibit the inflammatory response depending on the organ. Both limited and excessive vitamin A supplementation has been shown to ameliorate the intestinal inflammation by modulating the function of T cells (Kang, Wang et al. 2009). Treatment with retinoic acid reverses the emphysematous changes in a rat model of elastase-induced emphysema (Massaro and Massaro 1997). *All-trans* retinoic acid treatment

ameliorates inflammatory response in experimental autoimmune uveoretinitis and reduces Th1/Th17 responses (Keino, Watanabe et al. 2010). Supplementation of vitamin A increased the free radical production and increased mitochondrial electron transfer chain activity; however no pro-inflammatory activity has been reported in rat liver (de Oliveira, Soares Oliveira et al. 2009). Activation of retinoid X receptors by 9-*cis*-retinoic acid inhibits thymic stromal lymphopoietin (TSLP), which plays a key role in allergic airway inflammation. This effect is mediated via the effects of retinoic acid on the NF- κ B pathway (Lee, Headley et al. 2008). Treatment with retinoids significantly decreased neutrophil accumulation in mammary alveoli with reduced levels of TNF- α in mammary tissues and IL-8 in serum in a lipopolysaccharide-induced mastitis model in rats (Gu, Miao et al. 2009). Production of reactive oxygen species was also reduced following the treatment with retinoids (Gu, Zhu et al. 2009). *All-trans* retinoic acid ameliorates trinitrobenzene sulfonic acid-induced colitis by down regulating the inflammatory response. Ischemia-induced cerebral injury was reduced by *all-trans* retinoic acid by down regulating the cytokine production and inflammatory response (Choi, Kim et al. 2009). Systemic administration of retinoic acid reduced levels of pro-inflammatory cytokines after experimental spinal cord injury (van Neerven, Mey et al. 2010). Despite some conflicting data, it appears that retinoids have anti-inflammatory effects in various conditions.

1.15. Mechanism of anti-inflammatory effect

There have been only few studies regarding the mechanism of anti-inflammatory effect of vitamin A. One of the mechanisms is the suppression of proinflammatory gene transcription by retinoids through inhibition of the activity of transcription factors such as activator protein-1 (AP-1). Inhibition of AP-1 by retinoids leads to decreased production of inflammatory mediators such as collagenase, metalloproteinases (Thacher, Vasudevan et al. 2000). NF- κ B regulates the gene

transcription of proinflammatory cytokines and promotes lung inflammation induced by microbes or their products. Vitamin A pretreatment decreases the DNA binding activity of NF- κ B in the lung (Torii, Miyake et al. 2004). In addition, synthetic retinoids inhibit neutrophil recruitment (Norris, Osborn et al. 1987). Both AP-1 and NF- κ B are shown to be upregulated in asthmatic lung and induce a number of inflammatory genes. Therefore, negative regulation of both AP-1 and NF- κ B appears to be one of the major mechanisms of anti-inflammatory effect of vitamin A. In addition to the above mentioned mechanisms, retinoids have also been shown to affect toll-like receptor signaling that may contribute to their anti-inflammatory effects. *All-trans* retinoic (ATRA) acid down-regulates TLR2 expression and function in human monocytes and this may be one of the pathways of anti-inflammatory effect of retinoids. In the same study, they found that pretreatment with ATRA doesn't affect TLR4 expression, whereas co-treatment inhibits the TLR4-induced cytokine release such as IL-12, TNF- α and IL-6 (Liu, Krutzik et al. 2005).

1.16. Retinoids and the lung

Retinoids play a significant role in the development and function of lung. A sufficient and continuous availability of vitamin A is pivotal for a time-dependent regulation of the lung development (Zachman 1995). Vitamin A is essential for promoting normal epithelial differentiation and growth in respiratory tract (Biesalski and Stofft 1992). Together with glucocorticoids, retinoic acid regulates the formation of surfactant protein-A (Biesalski 2003). Deficiency of vitamin A causes keratinisation of respiratory tract epithelium and loss of ciliated cells, mucus, and goblet cells and is associated with lower respiratory tract infections (Tielsch, West et al. 1986; De Sole, Belay et al. 1987). Vitamin A deficiency also leads to emphysematous lungs, reduced lung elastin, decreased type II pneumocyte synthesis of surfactant and decreased

ornithine decarboxylase activity in pneumocytes (Baybutt, Hu et al. 2000). Retinoic acid signaling through its receptors is essential for the embryonic development of lung including alveologenesi. Overexpression of dominant negative RAR α causes alveolar abnormality with fewer alveoli and decreased surface area (Yang, Naltner et al. 2003). Furthermore, activation of RAR β induces expression of fibroblast growth factor Fgf10 and lung morphogenesis in early embryo. However, RAR α effects are also required to fine tune the effects mediated through RAR β (Desai, Chen et al. 2006). In addition to impaired lung development in vitamin A deficiency, the incidence of respiratory tract diseases is substantially increased, and recovery from the infections is hastened by the supplementation of vitamin A (Pinnock, Douglas et al. 1986; Sommer 1993). Utilization of vitamin A is increased during repeated allergen challenge and allergic bronchitis, most probably due to increased demand for epithelial repair (Shoseyov, Bibi et al. 2002). Low levels of vitamin A impair anti-oxidant capacity of lung resulting in increased susceptibility to development of emphysema in a cigarette smoke-induced model of emphysema (van Eijl, Mortaz et al. 2011). Endotoxin-induced chorioamnionitis decreases retinoic acid levels in fetal lungs in a sheep model. However, treatment with retinoic acid did not affect chorioamnionitis-induced fetal and systemic inflammation or IL-8 concentrations in lung tissue. Retinoic acid treatment also did not alter the lung structure (Kramer, Albertine et al. 2008). Taken together, retinoids play important roles in maintaining optimal lung physiology and health.

1.16.1. Retinoids and lung immunity

Retinoids also modulate lung immune response. Virus-induced airway hyperreactivity was inhibited by treatment with *all-trans* retinoic acid. This effect was attributed to both anti-inflammatory and antiviral mechanisms of retinoic acid (Moreno-Vinasco, Verbout et al. 2009).

Vitamin A supplementation ameliorates a number of inflammatory conditions of lung. Vitamin A administration caused a dose-dependent inhibition of ozone-induced neutrophilia, decreased airway hyperresponsiveness and caused a reduction in NF- κ B DNA binding activity (Hisada, Adcock et al. 1999). Vitamin A supplementation also caused a reduction in granulomatous changes, eosinophilic and neutrophilic infiltration into the lung, concentrations of TNF- α and eotaxin, and NF- κ B binding to DNA in Sephadex-treated rats (Torii, Miyake et al. 2004). The dietary supplementation of retinol reduced the vascular inflammation in lungs of monocrotaline treated rats (Swamidas, Basaraba et al. 1999). *We are not aware of any studies elucidate the role of vitamin A or retinoic acid in endotoxin-induced lung inflammation.*

CHAPTER 2: HYPOTHESES, OBJECTIVES, AND RATIONALE

2.1. HYPOTHESES

1. Retinoid receptors show differential expression in lungs across different species.
2. Expression of retinoid receptors is altered in acute lung inflammation.
3. Retinoic acid induces apoptosis in canine neutrophils.
4. Retinoic acid pre-treatment ameliorates endotoxin-induced acute lung inflammation.

2.2. OBJECTIVES

1. To characterize the expression pattern of retinoid receptors in lungs of cattle, pigs, and dogs.
2. To characterize the expression of retinoid receptors in normal and inflamed mouse and human lungs.
3. Determine the effects of retinoic acid on spontaneous apoptosis of resting and activated canine neutrophils.
4. To determine the effect of retinoic acid pre-treatment on endotoxin-induced acute lung inflammation.

2.3. RATIONALE

Lung inflammation is a severe pathological condition with high mortality. Neutrophils play a key role in pathogenesis of acute lung injury. The role of retinoids in lung development and function is well established. Retinoids also modulate immune response and possess anti-inflammatory. Effects of retinoids are mediated through retinoid receptors that belong to the family of nuclear receptor superfamily. Since retinoid receptors mediate effects of retinoids it is necessary to precisely understand their expression in both normal and inflamed lungs in various animal species such as cattle, dog, pig, human and mice to fully understand the role of retinoids.

Neutrophil apoptosis plays a key role in minimizing tissue damage and resolution of inflammation. Retinoids have an important role in regulation of programmed cell death of various cell types, and vitamin A deficiency has been related to impaired neutrophil apoptosis. Elucidation of roles of retinoids in neutrophil apoptosis may lead to development of therapeutic strategies to modulate neutrophil apoptosis. Therefore, because of the role of apoptosis in modulating inflammation, it is important to understand the effect of retinoic acid on apoptosis of both resting and activated neutrophils. Although retinoids have been shown to possess anti-inflammatory properties, no data are available on the effects of retinoids in endotoxin-induced acute lung inflammation. Because acute lung injury continues to elude new therapies, we sought to determine the effects of retinoic acid pre-treatment on acute lung inflammation.

CHAPTERS 3: EXPRESSION OF RETINOID RECEPTORS IN LUNGS OF CATTLE, DOG AND PIG

3.1. Abstract

Retinoids play important role in lung development and immune response. The effects of retinoids are mediated through two families of retinoid receptors; retinoic acid receptors (RARs) and retinoid X receptors (RXRs) with α , β , and γ subtypes in each family. To date no data exist on the expression pattern of retinoid receptors in lungs of cattle, dog and pigs. Therefore, we used western blotting, immunohistology, and immunoelectron microscopy to determine the expression of retinoid receptors in normal lungs of cattle, dog, and pigs (n=2 for each species). Western blot showed expression of all six retinoid receptor subtypes in pig lungs. Immunohistology data indicated differential expression of retinoid receptors in airway epithelium, vascular endothelium, alveolar/septal macrophages, and alveolar septum. Electron microscopy showed nuclear localization of retinoid receptors in neutrophils and pulmonary intravascular macrophages. RAR α subtype was localized in cytoplasmic vacuoles of pig monocytes. These data indicate constitutive expression of retinoid receptors in cattle, dog and pig lungs.

3.2. Introduction

The term 'retinoids' refers to natural and synthetic forms of vitamin A. Retinoids are essential for important functions such as vision, immune function, reproduction, maintenance of epithelium, and cellular differentiation (De Luca 1991). These compounds also have important roles in prenatal development and normal functioning of lung (Chytil 1992). Retinoic acid is the active metabolite of vitamin A, and it regulates gene expression during prenatal lung development and affects alveologenesis (Chytil 1996). Vitamin A deficiency leads to emphysematous lungs, reduced lung elastin, decreased synthesis of surfactant and reduced activity of ornithine decarboxylase in pneumocytes (Baybutt, Hu et al. 2000). Vitamin A

deficiency also causes keratinisation of respiratory tract epithelium, loss of ciliated cells and goblet cells and is associated with lower respiratory tract infections (Tielsch, West et al. 1986; De Sole, Belay et al. 1987).

The effects of retinoids are mediated through two families of nuclear receptors; retinoic acid receptors (RARs) (Linney 1992) and retinoid X receptors (RXRs) (Kliwer, 1994). Each family includes three subtypes, namely; α , β , and γ . Expression of retinoid receptors varies between the organs, and even within an organ the expression varies between different cell types. Deficiency of vitamin A leads to reduced expression of retinoic acid receptor subtypes α , β , and γ in lungs (Verma, Shoemaker et al. 1992). The mRNA levels of retinoic acid receptors are upregulated in lymph nodes of young children (Wei, Yang et al. 2007). Higher mRNA levels of RARs have also been reported during the prenatal development of mouse lung (Grummer, Thet et al. 1994). Retinoid receptor expression is altered in chronic inflammatory condition such as asthma and in neoplasms of lung (Soria, Xu et al. 2003; Brabender, Metzger et al. 2005; Druilhe, Zahm et al. 2008). Furthermore, expression of retinoid receptors also changes during the differentiation of both neutrophils (Taschner, Koesters et al. 2007), and monocytes (Fritsche, Stonehouse et al. 2000) and in activated neutrophils (Yost, Denis et al. 2004). Therefore, to fully understand the biology of retinoids and their cellular effects, it is important to precisely elucidate the expression of RAR and RXR subtypes in various cells and organs.

To date, no data are available on the expression of retinoid receptors in any domestic animal species. In addition to the veterinary medical importance of species such as cattle and pigs, species such as pigs and dogs are commonly used as animal models to study a variety of human lung diseases. Respiratory diseases such as Shipping Fever in cattle alone cause billions of dollars in losses to cattle industry in Canada and the USA (Ribble, Meek et al. 1995). Because

of the biomedical and economical implications, we designed this study to characterize the expression of retinoid receptor subtypes using western blot, immunohistochemistry and immunoelectron microscopy in normal lung tissues of cattle, dogs, and pigs.

3.3. Materials and methods

3.3.1. Reagents

Primary antibodies against all the six retinoid receptors were purchased from Santa Cruz Biotechnology, USA. Anti-human Von Willebrand Factor (vWF) and secondary antibodies conjugated with horse radish peroxidase for immunohistochemistry were obtained from Dako Corporation (Carpinteria, USA). Gold labelled secondary antibodies for immunoelectron microscopy were obtained from Jackson ImmunoResearch Laboratories, Inc (West Grove, USA). Color development kits and methyl green for counterstaining were purchased from Vector laboratories (Burlingame, USA). Pepsin and bovine serum albumin were obtained from Sigma Aldrich (St.Louis, USA).

3.3.2. Tissue processing

For immunohistochemistry, normal lung tissues were collected from cattle, dog, and pig (n=2 each) and fixed in 4% paraformaldehyde for 18 hours at 4°C. The tissues were dehydrated through increasing concentrations of alcohol, followed by clearing in xylene and embedded in paraffin. Sections of 5 µm thickness were cut and placed on polylysine-coated slides. The slides were kept at 60°C overnight to facilitate the adherence of tissue sections. For western blot, tissue samples were frozen in liquid nitrogen immediately after collection and stored at -80°C till further use. For immunoelectron microscopy, samples were fixed in 2% paraformaldehyde containing 0.1% glutaraldehyde for 3 hours at 4°C. They were dehydrated, infiltrated in epon/araldite, and embedded in resin (London resin company, USA).

3.3.3. Western blot analysis

Protein was extracted from frozen lung tissues using RIPA cell lysis buffer (50 mM Tris Hcl, 150 mM Nacl, 1 mM EDTA, 1% Triton X 100, 0.1% SDS, 1% Sodium deoxycholate, 1 mM PMSF, and 10 µl per ml protease inhibitor cocktail). The protein extract was mixed with 2X sample buffer and heated for 5 min in boiling water bath. For western blot analysis, samples were loaded on 12 % SDS-PAGE gel. Resolved proteins were transferred to nitrocellulose membrane (GE healthcare) and blocked with 5% skim milk powder in PBST (PBS plus 0.2% tween-20) to block nonspecific binding. The membrane was incubated with 1:100 concentration of rabbit anti-human antibodies to retinoid receptors at 4°C for overnight (Santa Cruz Biotechnology, San Diego, CA), followed by horseradish peroxidase conjugated goat anti-rabbit antibody (Dako, Canada). Detection was performed with the chemiluminescence detection reagents (GE healthcare) and experiment was repeated three times.

3.3.4. Immunohistochemistry

The immunohistochemistry protocols have been described previously (Schneberger, Lewis et al. 2011). Briefly, tissue sections were de-paraffinized in xylene and rehydrated with decreasing concentrations of alcohol followed by treatment with 0.5% hydrogen peroxide in methanol for 20 minutes to neutralize the endogenous peroxidase activity. Antigens were retrieved by treatment with 2mg/ml pepsin in 0.01N HCl for 45 minutes. The sections were incubated with 1% bovine serum albumin to prevent non-specific binding of antibody followed by incubation with primary antibody against retinoid receptors for 60 minutes at room temperature at the concentrations indicated in Table 1. The slides were washed with phosphate buffered saline 3 times, incubated with secondary antibody for 30 minutes and color development was done using peroxidase substrate kit from Vector Laboratories. Finally, tissue

sections were counterstained with methyl green, dehydrated, and mounted using Vectamount Permanent Mounting Medium (Vector Laboratories, Canada). For negative controls, primary antibody was omitted and sections were incubated with 1% bovine serum albumin.

3.3.5. Electron microscopy

For immuno-electron microscopy, 100 nm thick lung sections were first floated on blocking buffer (1% bovine serum albumin in Tris buffered saline) for 30 min and incubated with retinoic acid receptor antibodies at 1:10 dilution for 1 hour. Following three rinses of five minutes each with tris-buffered saline, the sections were treated with 20 nm gold-conjugated anti-rabbit secondary antibody for 1 hr and washed thrice with tris-buffered saline. The sections, counterstained with 2% uranyl acetate and lead citrate, were examined in a Philips 410LS transmission electron microscope. A negative control with omission of primary antibody was also run.

3.4. Results

3.4.1. Western blotting

Western blots on pig lung protein extracts detected bands of approximately 50-65kDa for all of the retinoid receptor subtypes examined in this study (Figure 3.1).

3.4.2. Immunohistochemistry controls

Lung sections from all species were stained with vWF antibody as a positive immunohistochemical control. Lung sections thus stained showed reaction on vascular endothelium (Fig.3.2A). In another control, the primary antibody was omitted and sections were incubated with relevant secondary antibodies. The incubation with only secondary antibodies resulted in lack of staining (Fig.3.2B).

3.4.3. Expression of retinoid receptors

The results on the expression of retinoid receptors in different cell types of lungs are summarized in Table 2. Because we examined expression of six different receptors subtypes in three species, we are showing only representative pictures of staining pattern of individual receptors subtypes in various cells in the lungs as described below.

3.4.3.1. Bronchial epithelium

Expression of RAR β and RAR γ was found in bronchial epithelium of all three species, (Figure 3.3A and B). In addition expression of all other subtypes (RAR α , RXR α , RXR β , and RXR γ) was found in bronchial epithelium of dog lungs (data not shown). Expression of RXR β was found in bronchial epithelium of both cattle and pigs (Figure 3.3C). Interestingly, although bronchial epithelium was negative for RXR α in pig lungs, smooth muscle surrounding the bronchial epithelium showed positive staining (3.3D).

3.4.3.2. Vascular endothelium

Vascular endothelium showed expression of RAR γ and RXR α in all three species (Figure 3.4 A and B). In addition, expression of RXR β and RXR γ was found in vascular endothelium of cattle lungs (Figure 3.4 C and D). However, dog lung vascular endothelium expressed all of the receptor subtypes except RXR γ . Vascular endothelium in pig lungs showed staining for RXR γ (Figure 3.4 E).

3.4.3.3. Alveolar/septal macrophages

Expression of RAR γ was found in alveolar/septal macrophages of all three species (Figure 3.5A). In addition, expression of RAR α was also found in alveolar/septal macrophages of both cattle and pig (Figure 3.5B). However, in pigs RXR β expression was also found in alveolar/septal macrophages (Figure 3.5C).

3.4.3.4. Alveolar septa

In cattle both RAR γ and RXR α expression was found in alveolar septa (Figure 3.6A). In contrast only RAR β expression was found in alveolar septa of dogs where as in pigs only RAR α expression was observed (Figure 3.6 B and C).

3.4.4. Immunoelectron microscopy

Immunoelectron microscopic analysis showed expression of RAR β in neutrophils of dogs (Figure 3.7A). RAR α was observed in monocytes in lungs of cattle and pig (Figure 3.7B). In pig monocytes RAR α was localized in cytoplasmic vacuoles. In addition, RAR γ and RAR β were expressed in pulmonary intravascular macrophages of cattle and pig, respectively (Figure 3.7C).

Table 1: Concentration of retinoid receptor primary antibodies for immunohistochemistry

Antibody	Cattle	Dog	Pig
RARα	1:50	1:50	1:100
RARβ	1:50	1:100	1:150
RARγ	1:100	1:100	1:150
RXRα	1:75	1:100	1:150
RXRβ	1:100	1:100	1:100
RXRγ	1:100	1:100	1:150

Table 2. Immunohistochemical localization of retinoid receptors in cattle, dog, and pig lungs.

	Cattle	Dog	Pig
RARα			
Bronchial epithelium	-	+	-
Vascular epithelium	-	+	-
Septal/alveolar macrophages	+	-	+
Alveolar septa	-	-	+
RARβ			
Bronchial epithelium	+	+	+
Vascular epithelium	-	+	-
Septal/alveolar macrophages	-	-	+
Alveolar septa	-	+	-
RARγ			
Bronchial epithelium	+	+	+
Vascular epithelium	+	+	+
Septal/alveolar macrophages	+	+	+
Alveolar septa	+	-	-
RXRα			
Bronchial epithelium	+	+	+
Vascular epithelium	+	+	+
Septal/alveolar macrophages	-	-	-
Alveolar septa	+	-	-
RXRβ			
Bronchial epithelium	-	+	+
Vascular epithelium	+	+	-
Septal/alveolar macrophages	-	-	+
Alveolar septa	-	-	-
RXRγ			
Bronchial epithelium	-	+	-
Vascular epithelium	+	-	+
Septal/alveolar macrophages	-	-	-
Alveolar Septa	-	-	-

+: positive staining, -: No staining

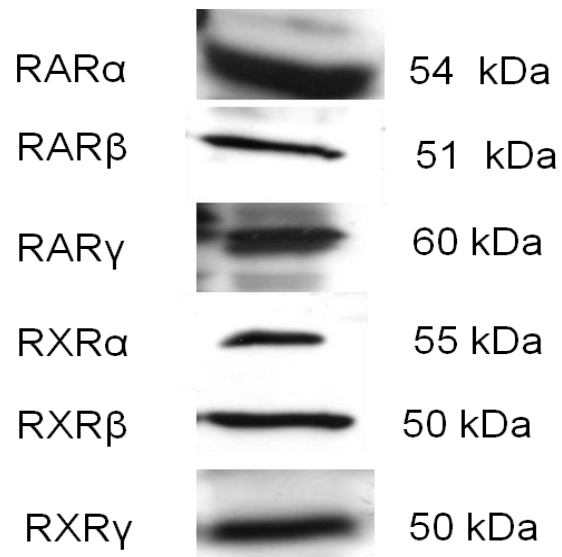


Figure 3.1: Western blot for retinoid receptors in pig lung. Protein extracts were isolated from pig lung tissue and run on a 12% polyacrylamide gel followed by incubation with retinoid receptor antibodies which detected bands of 50-65kDa .

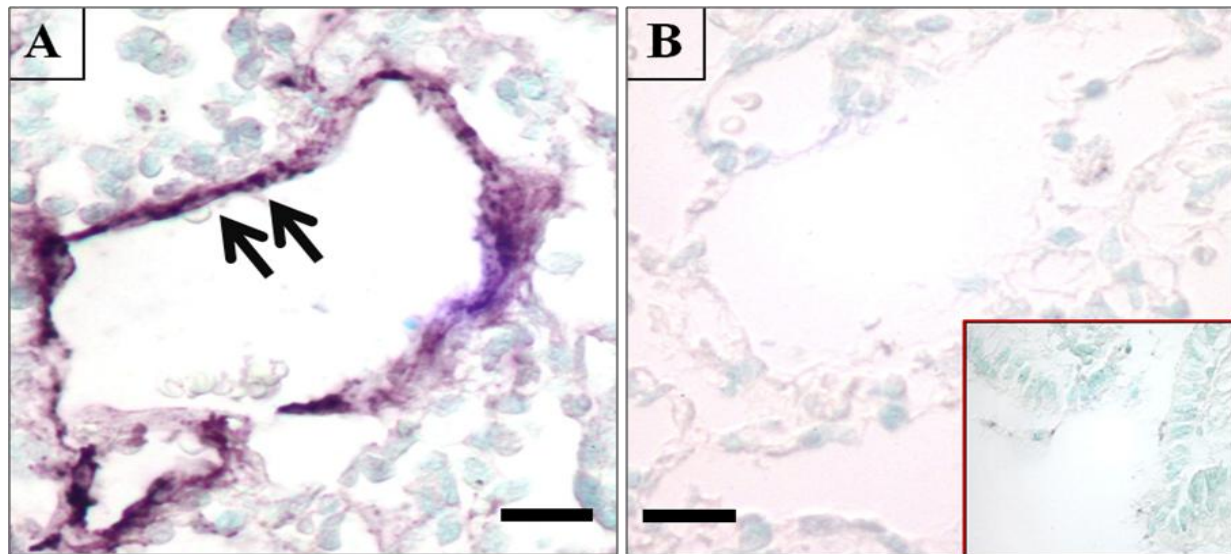


Figure 3.2. Positive and negative controls of immunohistochemical staining: Anti-vWF antibody stained only vascular endothelium (arrows) indicating the specificity of protocol (A) where as omitting primary antibody did not result in any staining (B). Original magnification A-B: 400X. Scale bars = 4 μ m.

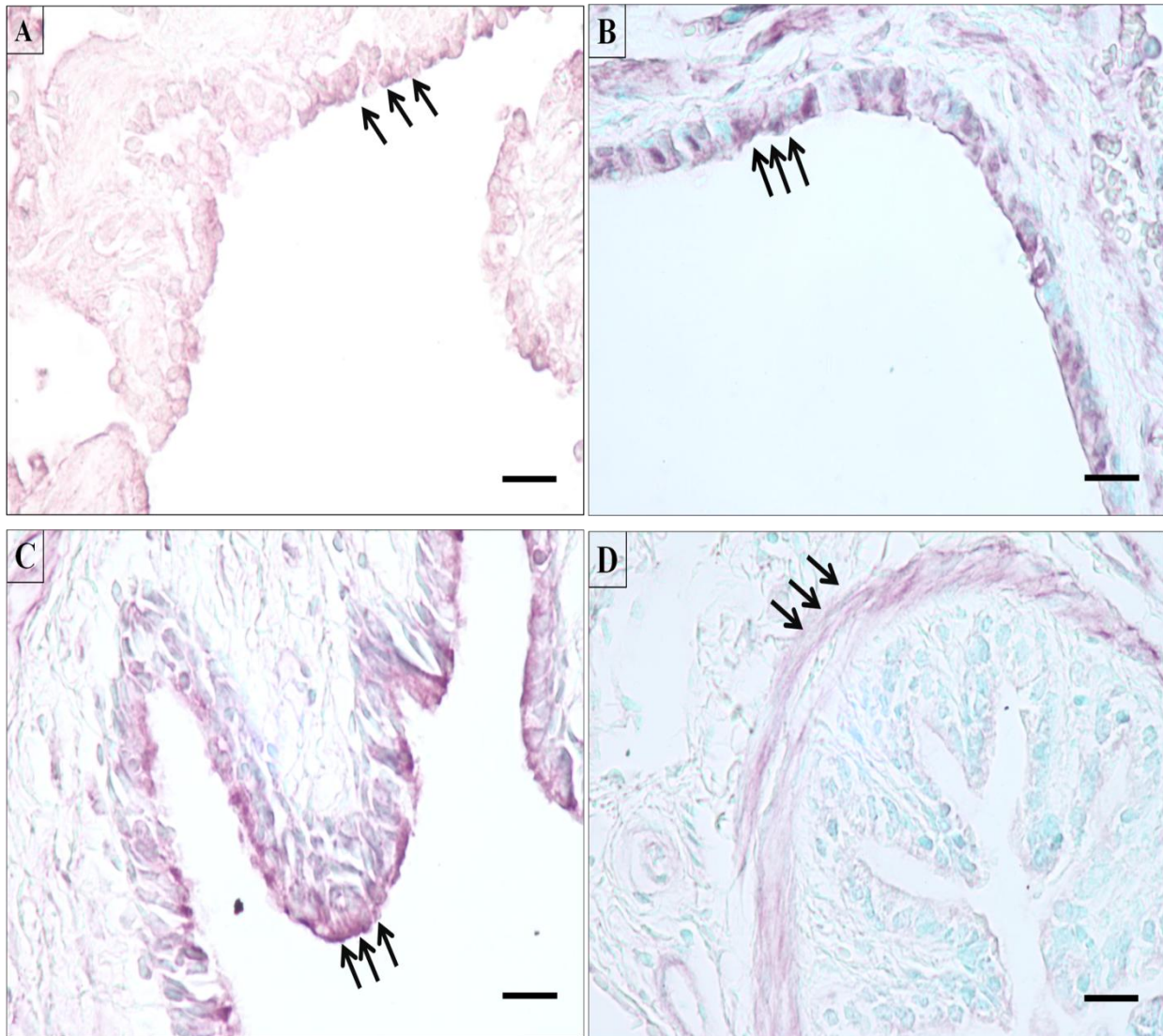


Figure 3.3: Lung immunohistochemistry for retinoid acid receptor subtypes in cattle, dog, and pig. Arrows indicate staining in bronchial epithelium for RAR β in dog (A), RAR γ in cattle (B), and RXR β in pig lungs (C). Expression was also found in smooth muscle surrounding airways for RXR α in pig lungs (D). Original magnification A-D: 400X. Scale bars = 4 μ m.

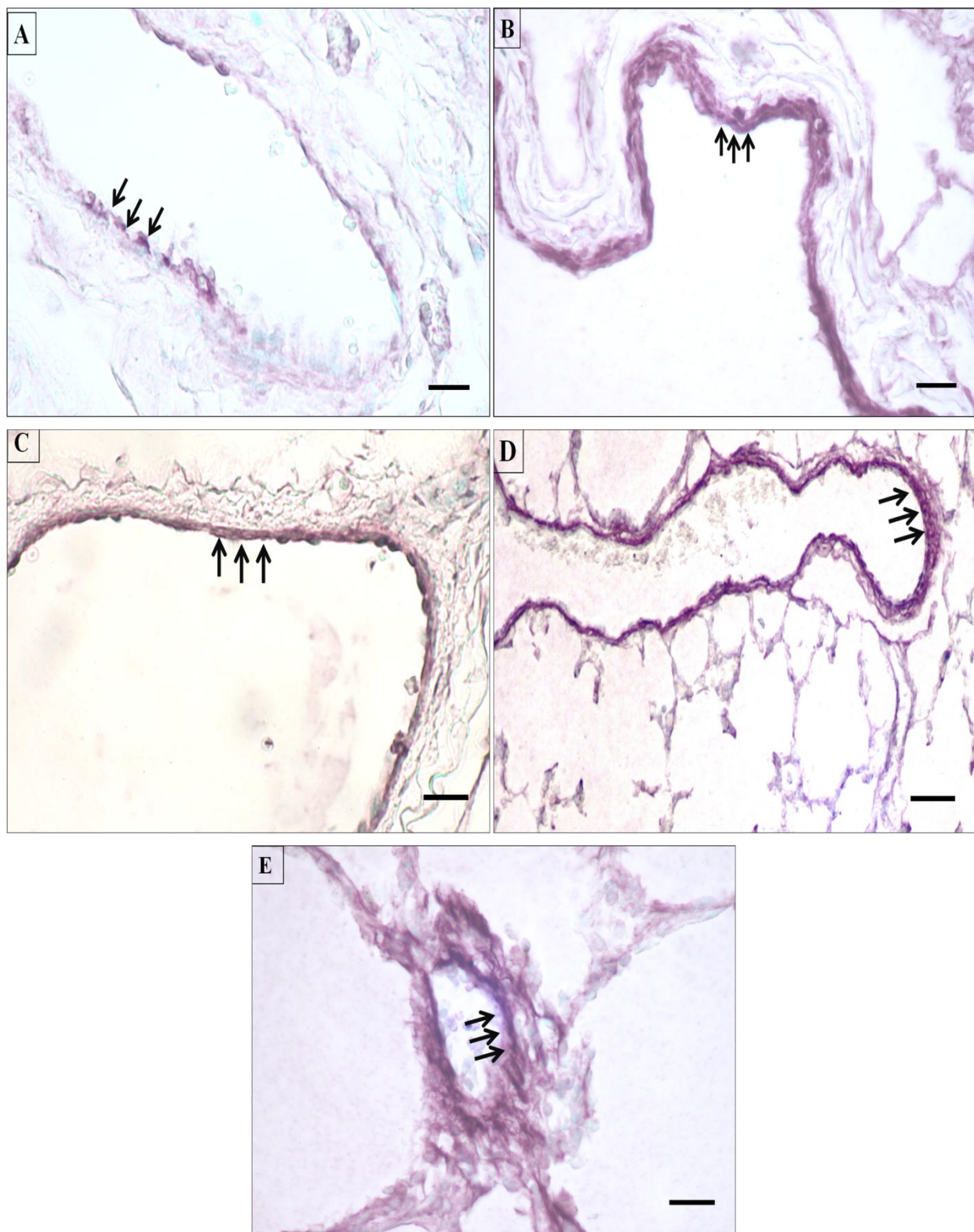


Figure 3.4: Lung immunohistochemistry for retinoid receptor subtypes in cattle, dog, and pig: Arrows indicate staining in vascular endothelium for RAR γ in cattle (A), RXR α in dog (B), RXR β (C), RXR γ (D) in cattle, and RXR γ (E) in pig lungs. Original magnification A-E: 400X. Scale bars = 4 μ m.

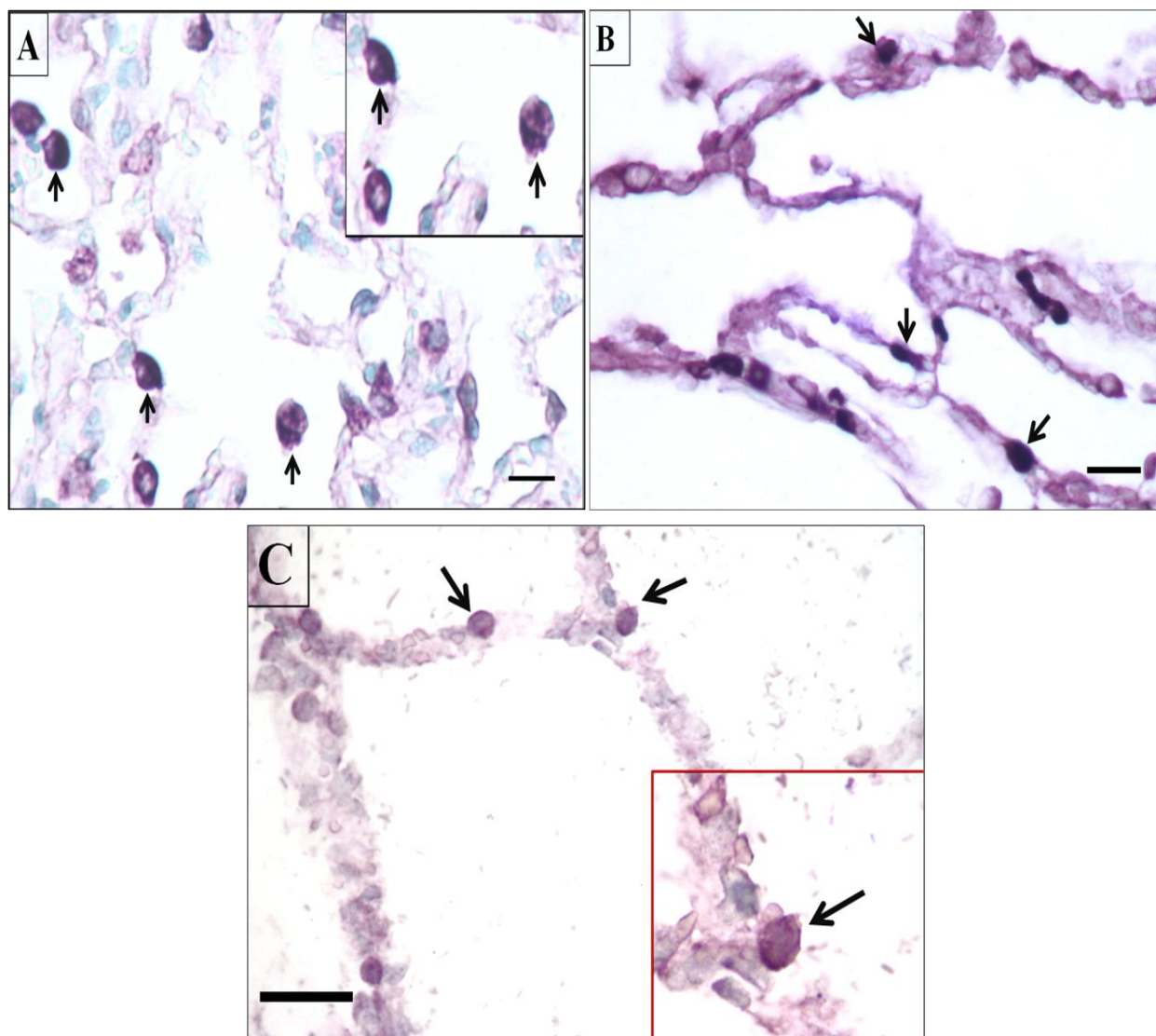


Figure 3.5: Lung immunohistochemistry for retinoid receptor subtypes in cattle, dog, and pig: Arrows indicate staining in alveolar/septal macrophages for RAR γ in dog (A), RAR α (B) and RXR β (C) in pig lungs. Original magnification A-C: 400X. Scale bars = 4 μ m.

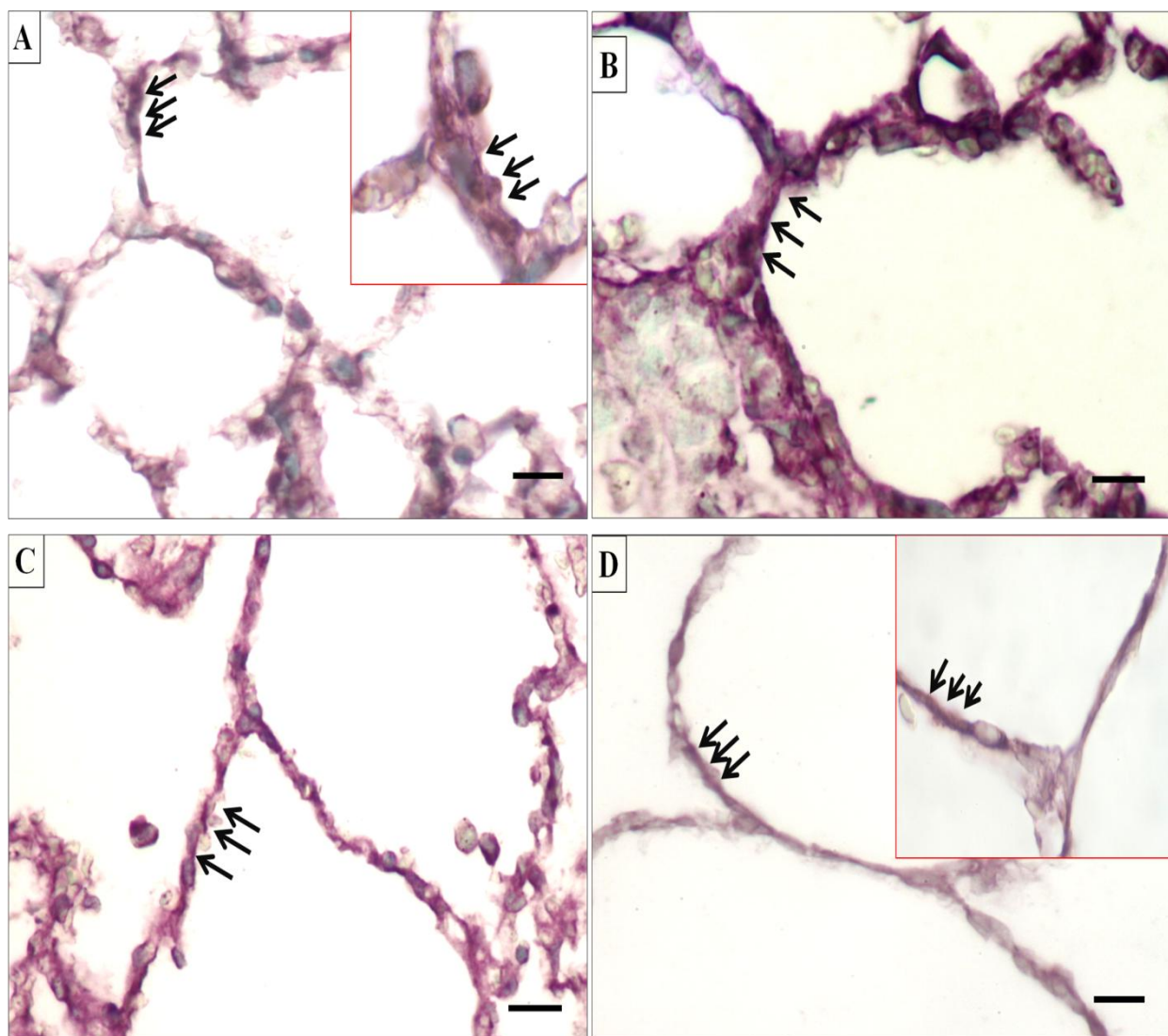


Figure 3.6: Lung immunohistochemistry for retinoid receptor subtypes in cattle, dog, and pig: Arrows indicate staining in alveolar septum for RAR γ (A) and RXR α (B) in cattle, RAR β in dog (C) and RAR α (D) in pig lungs. Original magnification A-D: 400 X. Scale bars = 4 μ m.

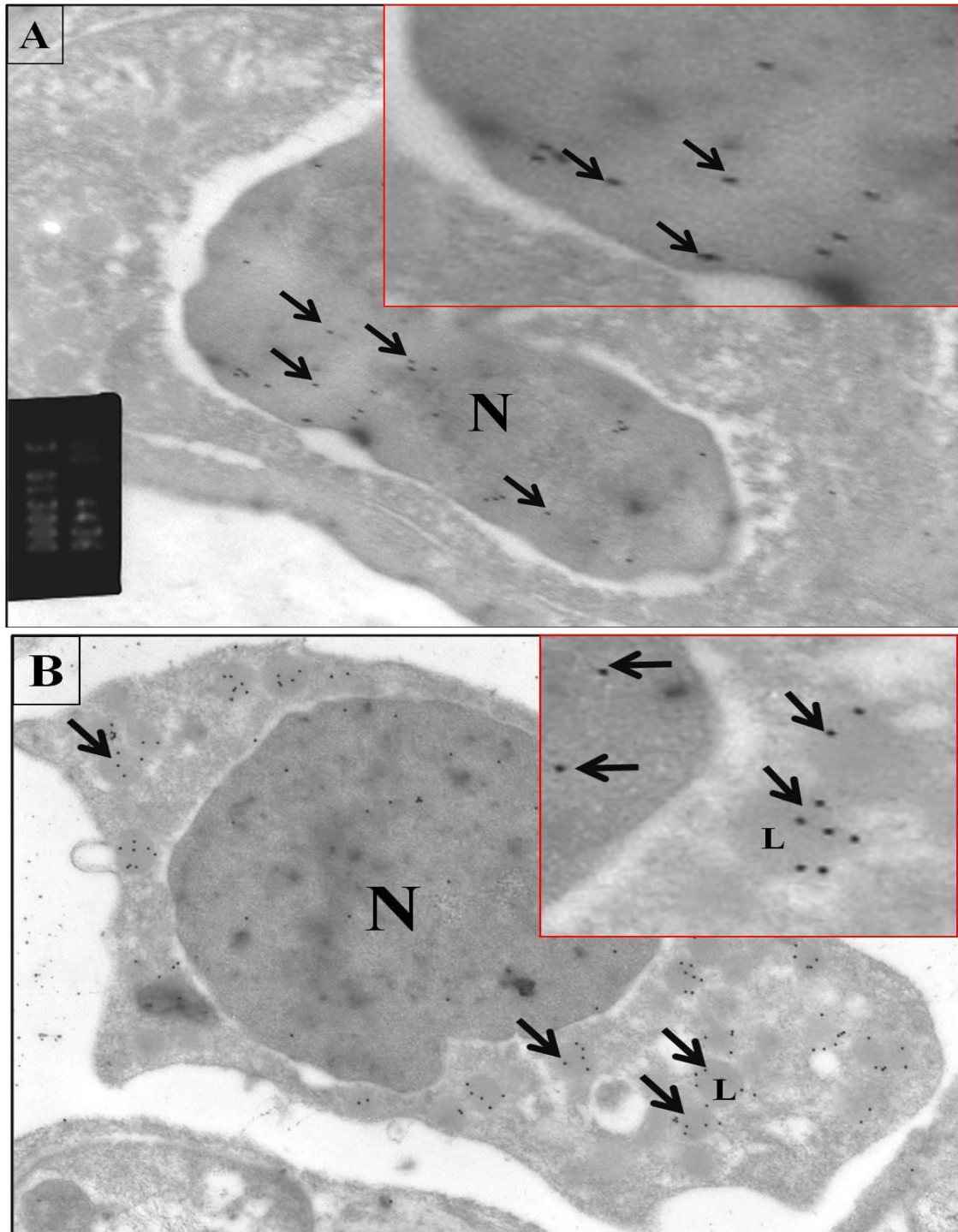


Figure 3.7: Immunoelectron microscopy for retinoid receptors: Labelling (arrows) for RAR β dog neutrophils (A) and for RAR α in monocytes of pig (B). N: Nucleus. L: Lysosome. Original magnification A-B: 13000X.

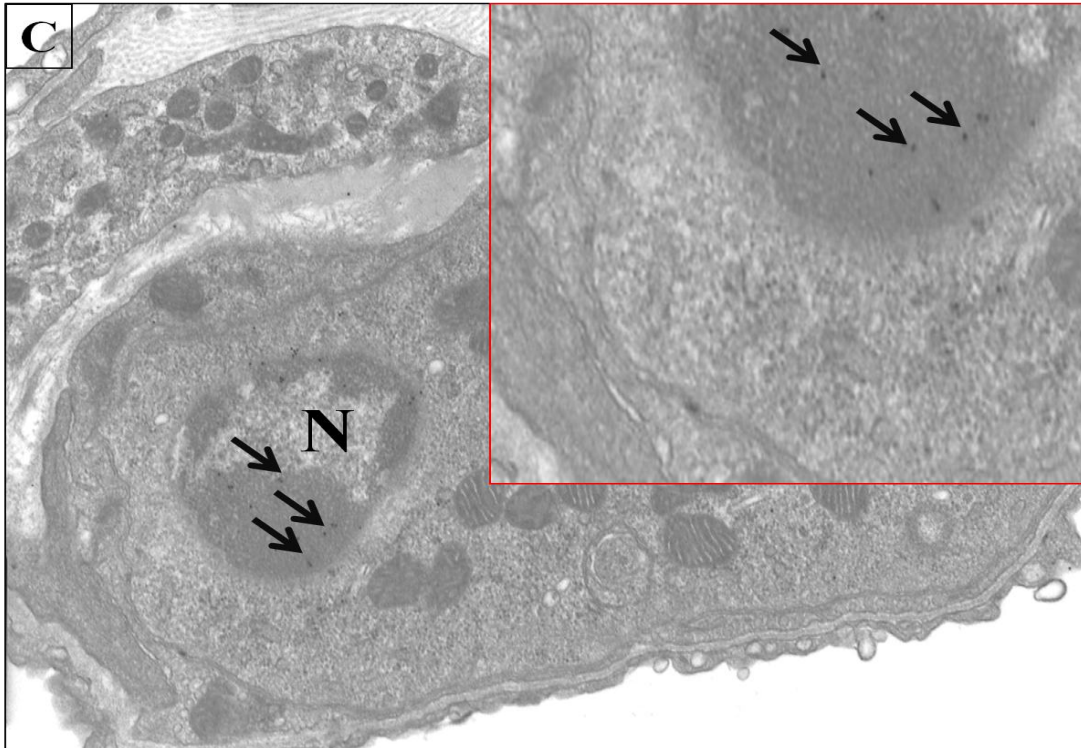


Figure 3.7: Immunoelectron microscopy for retinoid receptors: Labelling (arrows) for RAR γ pulmonary intravascular macrophages of cattle (C). N: Nucleus. Original magnification C: 13000X

3.5. Discussion

Retinoids play a key role in normal fetal lung development, function and immunity of the respiratory system. Effects of retinoids are mediated through two families of retinoid receptors; retinoic acid receptors and retinoid X receptors. Therefore, knowledge of expression pattern of retinoid receptors is important not only to understand the role of retinoids but also to correlate the altered expression with pathological conditions. Based on the previous studies, I hypothesised that retinoid receptors are differentially expressed between the species. Because no data are available on their expression in domestic animal species, I determined the expression of six retinoid receptor subtypes using western blot, immunohistology and immunoelectron microscopy.

Expression of retinoid receptors has been reported using both qualitative and quantitative techniques in human lungs and different laboratory animals. However, this is the first report on the expression pattern of retinoid receptors in domestic animal species. Because previous studies have shown differences in the expression of retinoid receptors not only between the species but also within each species (Hind, Corcoran et al. 2002; Rajatapiti, Kester et al. 2005), I examined receptor expression in three species of domestic animals. Our results also show that expression of retinoid receptors varies between different pulmonary cell types and results also show differential expression between the species. Differential expression indicates the possible distinct function of different retinoid receptor subtypes in specific cells. Bronchial epithelium was found to be expressing most of the retinoid receptor subtypes in cattle, dog, and pig lungs. This suggests their possible role in normal function and in pathological conditions involving airway epithelium. Retinoid receptors transduce the action of retinoic acid leading to formation of tight junctions in epithelial cells and maintenance of epithelial integrity (Kubota, Chiba et al. 2001;

Osanai, Nishikiori et al. 2007). The receptors also have a role in airway remodelling and regeneration in human asthma (Druilhe, Zahm et al. 2008). Retinoic acid has also been shown to reverse the airway hypersensitivity through its action on retinoid receptors (McGowan, Holmes et al. 2004). Therefore, the constitutive expression of various retinoid receptors in lung epithelium may be important for maintenance of epithelial barrier function and to prevent entry of pathogens.

Expression on vascular endothelium was detected for all the subtypes of RARs in cattle, RAR γ in dog, and RAR α , RAR β , and RXR β in pig. The role of lung vascular endothelium is important under normal and inflamed conditions. Acute lung inflammation is characterized by increased vascular permeability and development of lung edema (Wheeler and Bernard 2007; Maniatis and Orfanos 2008). Therefore, maintenance of adequate lung barrier capacity is critical for defense against deleterious effects of lung inflammation. It has been shown that retinoic acid inhibits vascular permeabilizing effect of vascular endothelial growth factor (Pal, Iruela-Arispe et al. 2000). Retinoic acid enhances antithrombotic potential of microvascular endothelial cells by inhibiting platelet tissue factor and increased expression of tissue plasminogen activator, which are important in maintaining the homeostasis (Marchetti, Vignoli et al. 2003). The data showing constitutive expression of retinoid receptors in lung endothelium underscores their physiological role in the maintenance of lung vascular barrier.

Alveolar and septal macrophages are the resident macrophages of lungs. Among the three species, pig lung showed positive staining for more receptor subtypes compared to calf and dog. Expression of RAR α was also detected in pulmonary intravascular macrophages (PIMs) of cattle lungs. Retinoids inhibit cytokine secretion from lipopolysaccharide stimulated monocytes and macrophages (Wang, Allen et al. 2007). Further studies are needed to elucidate the effects of

retinoids on macrophages in the context of pulmonary pathological conditions. Expression of retinoid receptor subtypes was also detected in monocytes of cattle and pig lung and dog neutrophils. Both monocytes and neutrophils play a crucial role in acute lung inflammation (O'Dea, Young et al. 2005; Grommes and Soehnlein 2011). Pulmonary intravascular macrophages are established as critical pro-inflammatory cells in the lungs of cattle and pig (Schneberger, Aharonson-Raz et al. 2012). PIMs promote recruitment of IL8-containing platelets in the inflamed lungs of cattle (Singh, Pearce et al. 2004). It is possible that the retinoid receptors expressed in PIMs may be targeted to reduce the pro-inflammatory potential of PIMs in the lungs of host species. Although PIMs are not present in normal human lungs, there is potential that PIMs are present in inflamed human lungs (Schneberger, Aharonson-Raz et al. 2012). If PIMs do develop in inflamed human lungs and promote inflammation, the retinoids may be used to curtail their activation and induction of pro-thrombotic phenotype in the endothelium. While the role of PIMs in human lungs is being clarified and we still believe that normal human lung lacks PIMs, the use of pig to model human lung inflammatory diseases remains questionable. Nevertheless, elucidation of function of retinoid receptor-signaling in these cells will help to better understand the role of retinoids in lung inflammation and open up possibilities for novel therapeutic strategies.

In conclusion, in the present study I found differential expression of retinoid receptors in lungs of cattle, dog, and pig and my hypothesis was proved. This might indicate distinct function of each receptor subtype in different cell types. Understanding the spatial expression of retinoid receptors is important in order to compare the altered expression in pathological conditions. Differential expression pattern can also have implications for gene transcription regulation by retinoids. Heterodimers between a RAR and RXR subtype is the functional unit that regulates

gene transcription (Thacher, Vasudevan et al. 2000). Differential expression can lead to formation of different heterodimers, subsequently leading to differential regulation of gene transcription. In addition, it also opens up the possibilities to use subtype specific ligands to minimise the adverse side effects. Further studies are needed to elucidate the role of retinoid receptor-signaling pathways in pulmonary pathobiology in domestic animal species. The implications of expression of retinoid receptors in PIMs for lung pathophysiology and their contribution to total expression of retinoid receptors in the lung needs to be studied. Lastly, it would also be important to study the mRNA expression of various receptors in normal and inflamed lungs.

CHAPTER 4: CHAPTER 4: EXPRESSION OF RETINOID RECEPTORS IN MURINE ACUTE LUNG INJURY AND INFLAMED HUMAN LUNGS

4.1. Abstract

Retinoid receptors are members of nuclear receptor superfamily consisting of two distinct families: RARs (retinoic acid receptors) and RXRs (retinoid X receptors). Each family contains three receptor subtypes α , β , and γ . Retinoids transduce their effects through binding to retinoid receptors and inhibit transcription factors such as activator protein-1 and nuclear factor- κ B (NF- κ B) both of which regulate the transcription of several inflammatory genes. Considering the role of retinoid receptors in lung physiology, we need a precise understanding of their expression in normal and inflamed lungs. We used immunohistochemistry and Western blot to determine the expression of retinoid receptors in a murine model of endotoxin-induced acute lung inflammation and inflamed human lungs. Western blot showed expression of all six retinoid receptor subtypes in normal and inflamed mouse lungs. Immunohistology localized differential expression of retinoid receptors in airway epithelium, alveolar/septal macrophages, vascular endothelium and alveolar septum in mouse lungs. Intranasal LPS (*E. coli*; 055:B5, 80 μ g) challenge in mice resulted in increased expression of RXR α in airway epithelium compared to control animals. Although expression of all the six retinoid receptor subtypes was found in normal human lungs, we did not find any differences in expression between normal and inflamed human lungs. Immunoelectron microscopy showed nuclear localization of RAR γ in alveolar macrophages of both human and mouse lungs and human neutrophils. The basal and altered expression of retinoid receptors in normal and inflamed lungs, respectively, suggests their underlying role in lung pathophysiology.

4.2. Introduction

Retinoids include natural vitamin A and its synthetic derivatives. They are required for a wide variety of biological functions including optimum functioning of the immune system (Thacher, Vasudevan et al. 2000). The retinoids exert their cellular effects through two families of nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), both of which belong to nuclear receptor superfamily (Giguere, Ong et al. 1987). The heterodimers between RAR and RXR receptor subtypes regulate retinoid-mediated gene transcription. RXRs also form homodimers or heterodimers with other nuclear receptors (Chambon 1996). Both *all trans* retinoic acid and 9-cis-retinoic acid act as ligands for RARs where as only 9-cis-retinoic acid binds to RXRs (Stunnenberg 1993). The role of retinoids in lung development and function is well established. Deficiency of retinoids leads to keratinizing metaplasia of columnar epithelium in respiratory tract (Chytil 1996). Retinoic acid supplementation reverses increased airway hypersensitivity (McGowan, Holmes et al. 2004). Therefore, retinoids through their binding to retinoid receptors appear to have a major role in lung physiology.

Acute inflammation accompanies many lung diseases characterized by diffuse damage to lung parenchyma and it has been associated with high mortality (Dushianthan, Grocott et al. 2011). The mortality rates of patients suffering from acute lung injury associated with diseases such as acute respiratory distress syndrome is reported to be 35 to 40% (Rubinfeld and Herridge 2007). Pathogenesis of acute lung injury is complex because many cells such as activated neutrophils, alveolar macrophages, and endothelial cells play important roles. Alveolar macrophages regulate recruitment and fate of neutrophils at the site of inflammation. Activated neutrophils recruited in response to microbial products and chemokines secreted by alveolar macrophages, though necessary for host defense, they do cause considerable tissue damage

(Segel, Halterman et al. 2011). Vascular endothelial cells facilitate neutrophil migration to inflammatory sites and also become leaky in acute lung inflammation. Vitamin A deficiency has been shown to exacerbate inflammatory response through activation of transcription factors such as NF- κ B (Reifen, Nur et al. 2002). Vitamin A deficiency resulted in increased expression of Toll like receptor-2 and MyD88 in mucosal surfaces (Yang, Yuan et al. 2011). Expression of retinoid receptors is affected in a number of pathological conditions. The epithelial expression of RAR γ , RXR α , and RXR γ was increased in severe asthma compared to patients with milder disease and to control subjects (Druilhe, Zahm et al. 2008). Taken together, it is believed that retinoids and their receptors play important roles in normal and inflamed lungs.

Currently, there are only a few data, if any, on the expression of retinoid receptors in normal and inflamed lungs. Since lung cells such as alveolar macrophages, vascular endothelium and neutrophils coordinate to generate an inflammatory response in various conditions such as asthma and acute lung respiratory distress syndrome, it is important to understand the expression of retinoid receptors in these and other cells in the lung. To localize cell-specific expression of various retinoid receptors, I studied their expression in intact lungs and not isolated cells. Therefore, the objective of this study was to characterize the expression of RAR and RXR subtypes in normal mice and human lungs and to compare expression pattern between normal and inflamed lungs.

4.3. Materials and Methods

4.3.1. Animals

Male C57BL/6 mice, 8 to 10 weeks of age were used and experiments were conducted in accordance with local ethics committee guidelines. Animals were given standard laboratory diet and had free access to water.

4.3.2. Experimental design

Three time points (6, 12, and 24 hours) were used to determine the expression of retinoid receptors in inflamed lungs. Six groups (n=5 per group) of mice were used and for each time point there was a saline and LPS-treated group. Mice were treated with 80 µg lipopolysaccharide (*E. coli*, 055:B5) in 80 µl endotoxin free saline or with saline (80 µl) by intranasal route. At the above indicated time points following the treatment, bronchoalveolar lavage was done and animals were euthanized to collect lung tissues for further analysis.

4.3.3. Human lung samples

Human lung samples were obtained following the consent from the archives of the Department of Pathology in the College of Medicine at the University of Saskatchewan. The inflamed human lungs (n=3) were collected from patients died because of sepsis. The sepsis cases satisfied the criteria set for the clinical definition of sepsis (Riedemann, Guo et al. 2003). Normal lung samples (n=3) were collected from those with no apparent clinical conditions and lung pathology. Tissue sections were cut from paraffin embedded sections and placed on poly-L-lysine coated glass slides.

4.3.4. Tissue processing

Lung tissues for western blotting were immediately frozen in liquid nitrogen and stored at -80°C till further analysis. Lungs for immunocytochemistry were fixed in 4% paraformaldehyde at 4°C for overnight. The lungs were then dehydrated through a graded series of alcohol solutions, cleared in xylene and embedded in paraffin. Five µm sections were cut and mounted on polylysine-coated slides.

4.3.5. Western Blotting

Proteins were extracted from mouse lungs using RIPA cell lysis buffer (50 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X 100, 0.1% SDS, 1% Sodium deoxycholate, 1 mM

PMSF, and 10 µl per ml protease inhibitor cocktail) and the proteins were separated on a 12% polyacrylamide gel and transferred on to Hybond-ECL nitrocellulose membrane (GE Bioscience, Germany). Membranes were then blocked with 5% skim milk in phosphate buffered saline (PBS ; pH 7.4) and incubated with retinoid receptor antibodies (Santa Cruz Biotechnology, USA) for overnight at 4°C. Membranes were then washed and incubated for 1 hour with goat anti-rabbit HRP (Dako, Ontario, Canada). Blots were developed using the EC Western Blotting Detection kit (GE Bioscience, UK) and exposed to Hyperfilm ECL film (GE Bioscience, UK).

4.3.6. Immunohistology

Immunohistology was done using retinoid receptor antibodies on both mouse and human lung tissue sections. Briefly, tissue sections were de-paraffinized, re-hydrated, and tissue peroxidase activity was quenched (0.5% hydrogen peroxide in methanol). Sections were then treated with pepsin (2mg/ml 0.01N hydrochloric acid) for antigen unmasking and blocked with 1% BSA for 30 mins to block non-specific binding. Sections were incubated with retinoid receptor antibodies (The concentrations indicated in Table 1) for 1 h at room temperature. The sections were then washed with phosphate buffered saline and incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (Dako, Ontario, Canada) at 1:150 dilution for 30 min at room temperature. Color developing kit (Vector Laboratories, Ontario, Canada) was used for color development. Slides were counterstained with methyl green (Vector Laboratories) and mounted using permanent mounting medium. For negative control, primary antibody was omitted and for the positive control, sections were incubated with anti von Willebrand factor (vWF). Alveolar/septal macrophages positively stained for retinoid receptors were counted in five randomly selected fields in lung sections from saline-treated animals (N=4) at 400X magnification.

4.3.7. Immuno-electron Microscopy

For immuno-electron microscopy, thin lung sections were placed on nickel grids and floated in blocking buffer for 30min prior to 1hr incubation with retinoic acid receptor antibodies. Sections were rinsed 3 times in Tris-buffered saline for 5 minutes before addition and incubation with 20 nm gold-conjugated anti-rabbit secondary antibody (Fitzgerald Industries International, Concord, MA, USA) for 1hr. A control with omission of the primary antibody was also run.

4.3.8. Statistical Analysis

Data were presented as mean \pm SE. Statistical analysis was done by one-way ANOVA using GraphPad prism software and $p \leq 0.05$ was considered statistically significant.

4.4. Results

4.4.1. Western blotting

In order to determine the expression of retinoid receptors in mouse lungs, Western blotting was done using antihuman antibodies against retinoid receptors known to cross-react with mouse retinoid receptors. Western blots of protein extracts from mice showed bands of approximately 50-65 kDa, (Figure 4.1 and 4.2-A). Densitometry showed a significant increase in the expression levels of RXR α in lung extracts of LPS-treated animals compared to saline treated counterparts (Figure 4.2- A and B). But there were no differences in the expression of RXR α between the LPS-treated groups. Furthermore, there were no differences in the protein expression of other subtypes in lungs of normal and LPS-treated mice (data not shown).

4.4.2. Immunohistochemistry

As a positive control for immunohistochemistry, sections were incubated with anti-vWF antibody which only stained vascular endothelium (Fig.4.3-A). For the negative control, primary

antibody was omitted and sections were incubated with only secondary antibody. This resulted in no staining (Fig.4.3-B).

4.4.2. Expression in normal and inflamed mouse lungs

The cell-specific expression of various retinoid receptor subtypes was explored by staining the lung tissues with the relevant antibodies. The data on the expression of individual receptor subtypes in lungs from normal mice are summarized in Table 2. I am also including some examples of immunohistochemical staining of specific retinoid receptors. Figure 4 shows expression of RAR α (Figure 4.4-A) and RAR β (Figure 4.4-B) in vascular endothelium and airway epithelium in the mouse lung. RAR γ was localized in alveolar macrophages and alveolar septum (Figure 4.5). The counts of alveolar macrophages stained for various retinoid receptors showed that the highest number were positive for RAR γ (Figure 4.6).

Lung tissues from the LPS-treated mice were probed for the expression of various retinoid receptors. An example of LPS-induced alteration in the expression of retinoid receptors is shown in Figure 4.7. The staining intensity of RXR α was increased in the LPS-treated mouse lungs compared to normal lung (Figure 4.7). Western blots coupled with densitometry confirmed increased protein levels of RXR α in LPS treated animals (Figure 4.2-A and B).

4.4.3. Expression of retinoid receptors in human lungs

Mouse is the most commonly used laboratory animal model to study human diseases. However, to obtain more precise data on the expression of retinoid receptors in human lungs, we performed immunohistochemistry on normal and septic human lungs. The data on the expression of various receptors in normal human lungs are summarized in Table 3. Figure 8 shows representative pictures of the staining pattern of RXR γ in bronchial epithelium (Figure 4.8-A), RAR α vascular endothelium (Figure 4.8-B), and RAR γ in alveolar macrophages (Figure 4.8-C).

We did not find any differences in the number of positive cells for retinoid receptor subtypes between normal and septic human lungs (data not shown).

4.5. Immunoelectron microscopy

To elucidate the subcellular localization of retinoic acid receptors in different cell types of mouse and human lungs, we performed immunoelectron microscopy. Expression of RAR γ was found in the nuclei of both human (Figure 4.9-A) and mouse (Figure 4.9-B) alveolar macrophages. The data also showed RAR γ in nuclei of human neutrophils (Figure 4.10).

Table 1: Concentration of retinoid receptor primary antibodies for immunohistochemistry

Antibody	Mouse	Human
RARα	1:50	1:50
RARβ	1:50	1:125
RARγ	1:125	1:150
RXRα	1:125	1:100
RXRβ	1:100	1:100
RXRγ	1:100	1:100

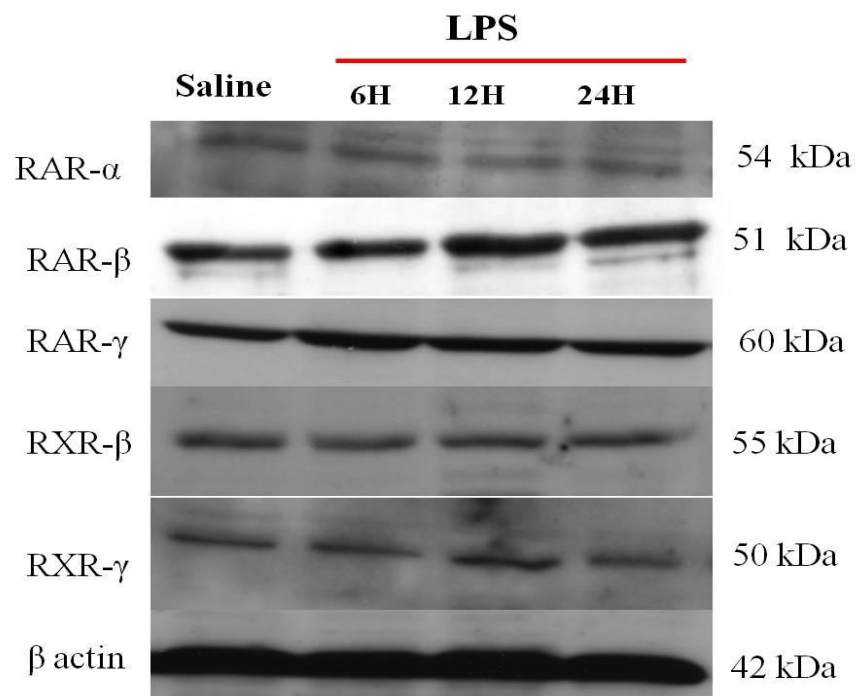
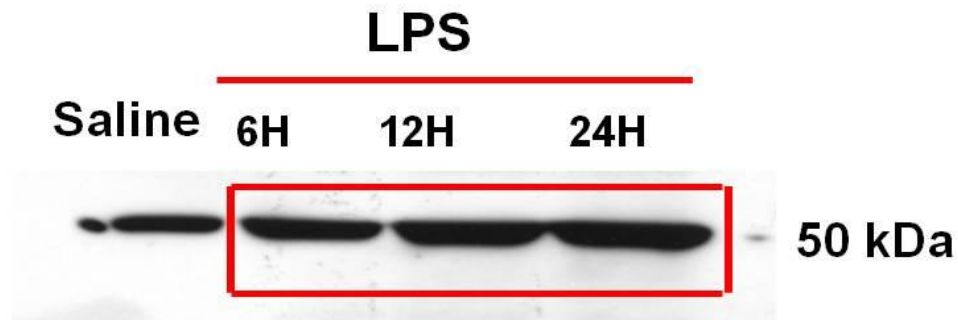


Figure 4.1: Western blot analysis for retinoid receptors in protein extract of control and LPS-treated mice. Representative blots from lungs of control and LPS-treated animals.



B

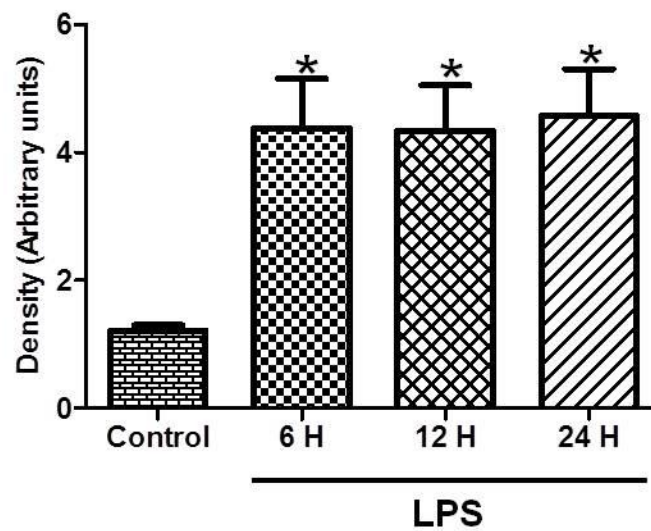


Figure 4.2. Western blot analysis for RXR α in protein extract of control and LPS-treated mice. A. Representative blot from lungs of control and LPS-treated animals. B. Densitometric plot shows protein levels in lungs from control and LPS-treated mice. Data are presented as mean \pm SE of three animals from each group.

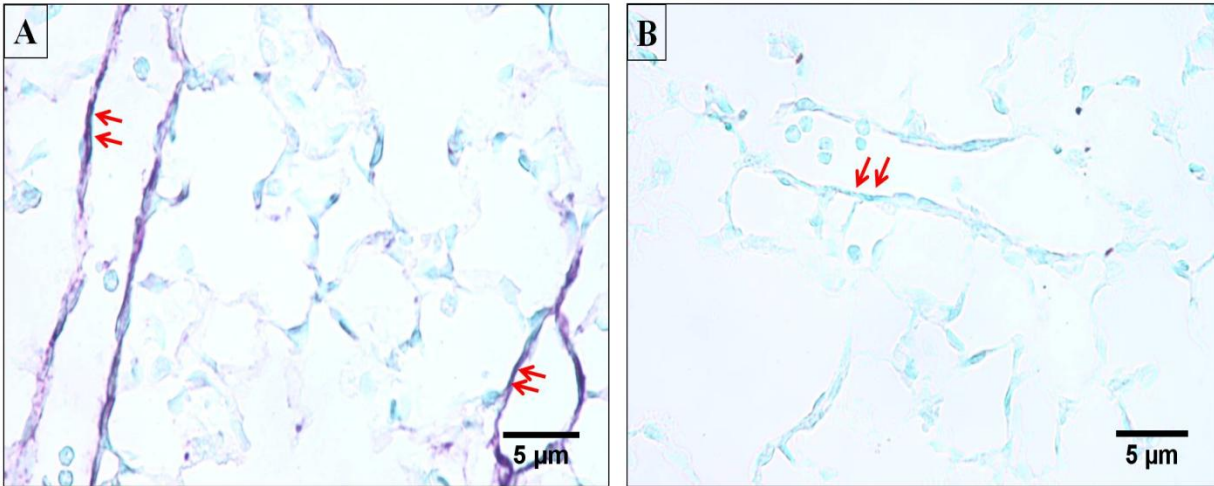


Figure 4.3. Immunohistochemical controls. Staining with vWF antibody was localized only in vascular endothelium (arrows) indicating the specificity of the protocol (A) where as omitting primary antibody did not result in any staining (B). Original magnification A-B: 400X

Table 2. Immunohistochemical localization of retinoid receptors in mouse lung

	Bronchial epithelium	Vascular endothelium	Alveolar/septal macrophages	Alveolar epithelium	Effect of LPS treatment
RARα	-	+	+	-	No change
RARβ	+	+	-	-	No change
RARγ	+	+	+	+	No change
RXRα	+	-	-	-	↑
RXRβ	+	-	+	-	No change
RXRγ	+	+	+	-	No change

+•: positive staining, -: Negative staining.

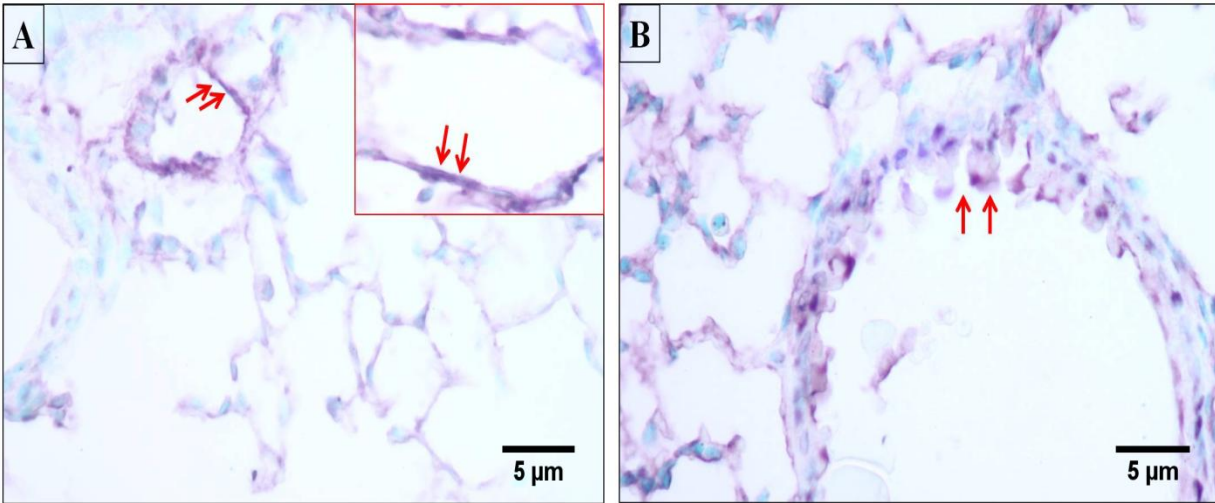


Figure 4.4. Immunohistochemical localization of retinoid receptors in vascular endothelium and bronchial epithelium of normal mouse lungs. Arrows show positive staining for RAR α in vascular endothelium (A) and RAR β in bronchial epithelium (B). Original magnification A-B: 400X

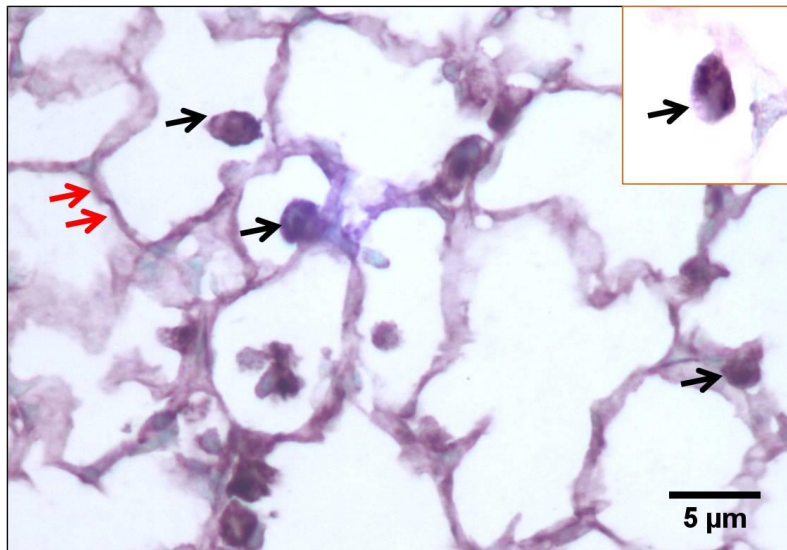


Figure 4.5. Immunohistochemical localization of retinoid receptors in mouse lungs. Red arrows indicate expression of RAR γ in alveolar epithelium and black arrows indicate expression of RAR γ in alveolar/septal macrophages. Original magnification: 400X

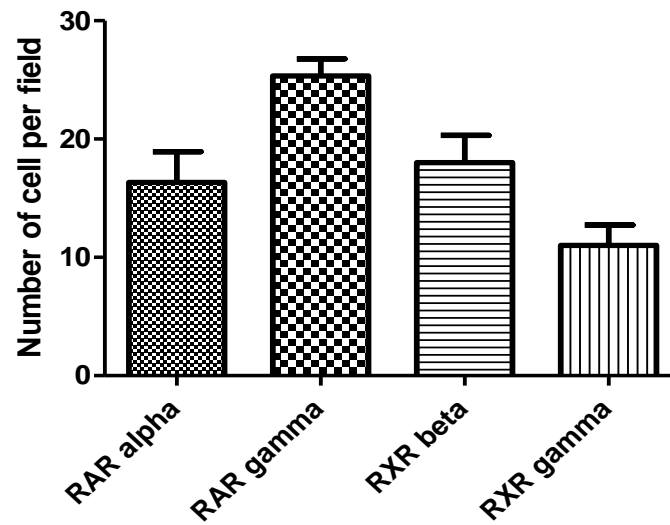


Figure 4.6. Relative expression of retinoid receptors in alveolar/septal macrophages of normal mouse lungs. Positive alveolar/septal macrophages were counted from 5 randomly selected fields from 3 animals. Results are presented as mean \pm SE.

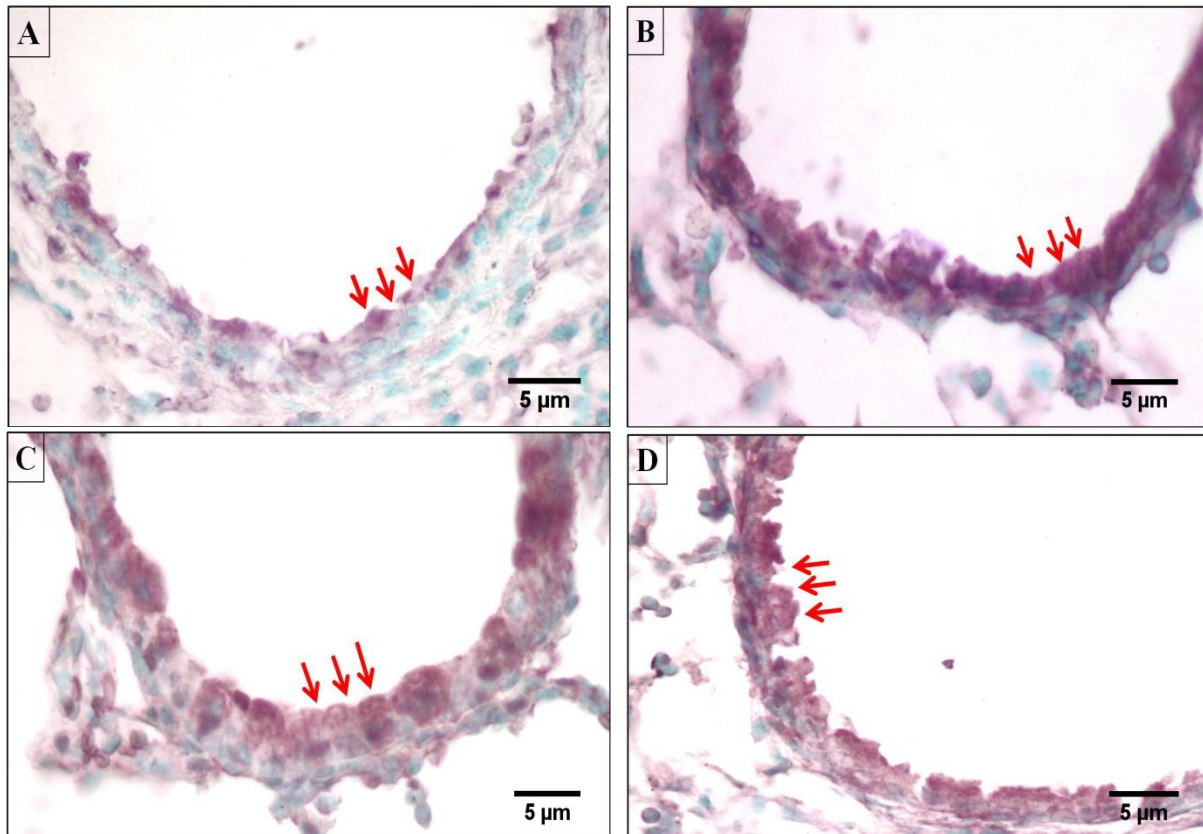


Figure 4.7. Immunohistochemistry for RXR α in mouse lungs. Lung sections from control (A) and LPS treated (6 H - B, 12 H - C, 24 H - D) staining for RXR α in airways. Original magnification A-D: 400X.

Table 3. Immunohistochemical localization of retinoid receptors in normal human lungs

	Bronchial epithelium	Vascular endothelium	Alveolar/septal macrophages	Alveolar epithelium
RARα	-	+	-	-
RARβ	-	+	+	-
RARγ	+	+	+	+
RXRα	+	-	-	+
RXRβ	+	-	+	-
RXRγ	+	+	+	-

+: Positive staining, -: No staining.

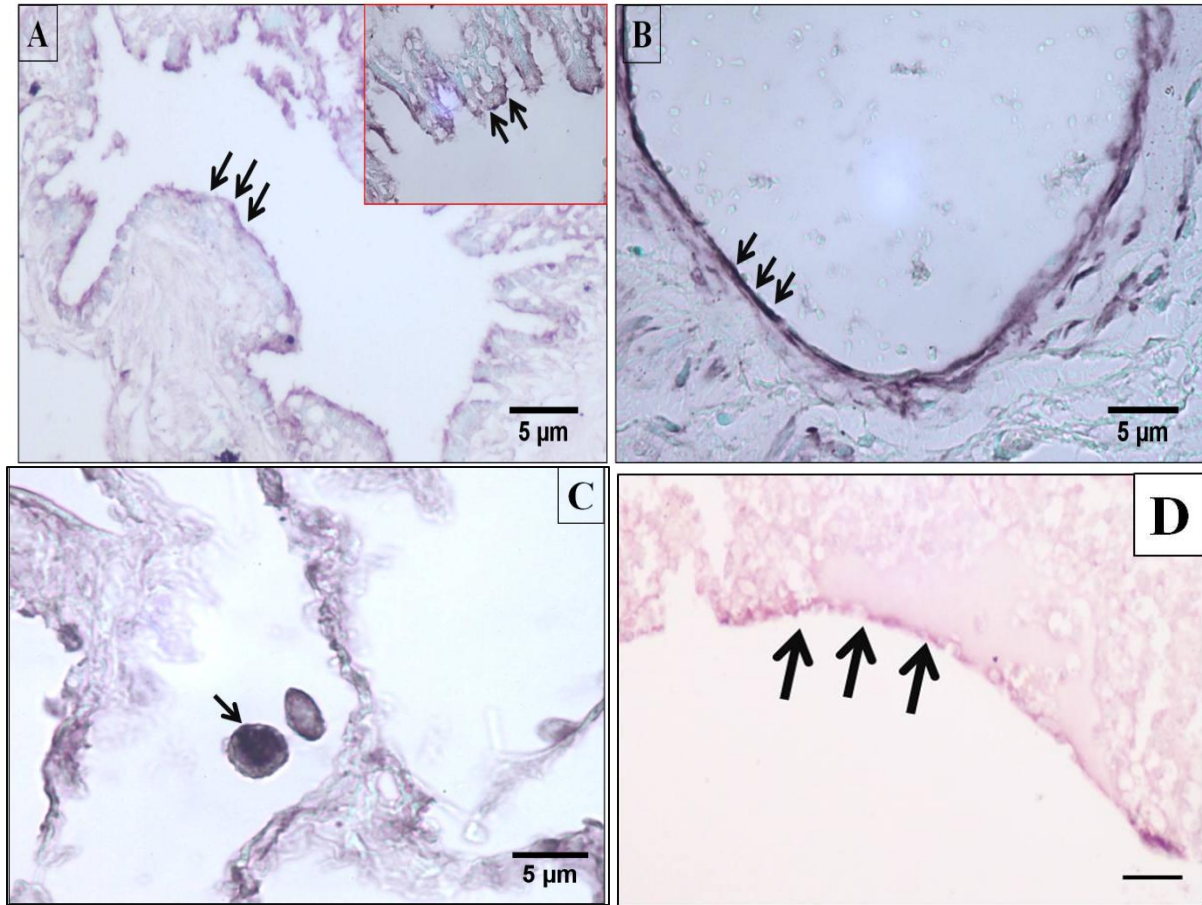


Figure 4.8. Immunohistochemical localization of retinoid receptors in normal and inflamed human lungs. Arrows indicate expression of RXR γ in bronchial epithelium (A), RAR α in vascular endothelium (B) and RAR γ in alveolar macrophages (C) of normal human lungs. Figure D shows staining of vascular endothelium for RAR α in inflamed human lungs. Original magnification A-D: 400X

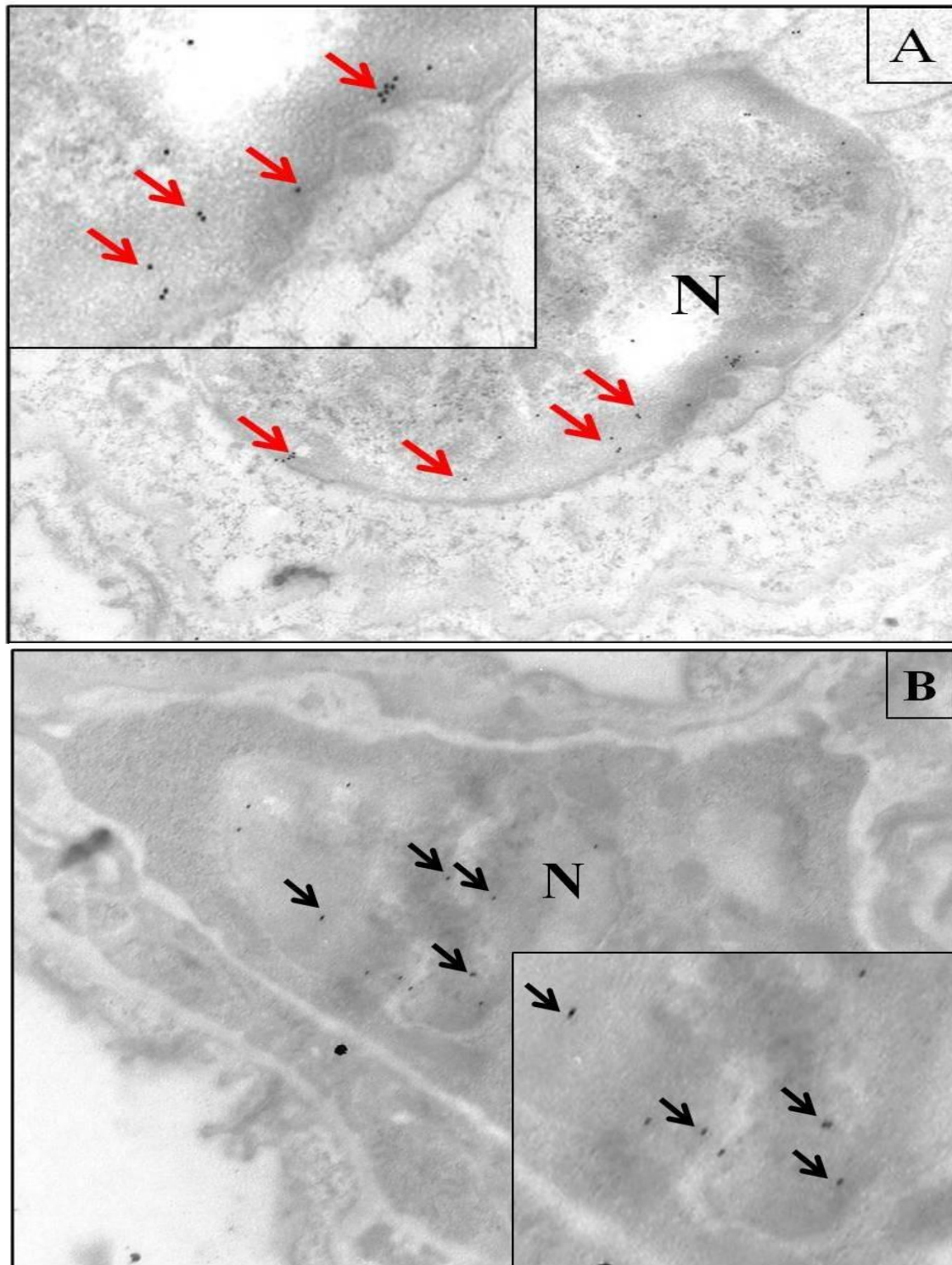


Figure 4.9. Immunoelectron microscopy for retinoid receptors in normal lungs. Arrows show labelling for RAR γ in human (A) and mouse (B) alveolar macrophages. N: Nucleus. Original magnification A-B: 13000X

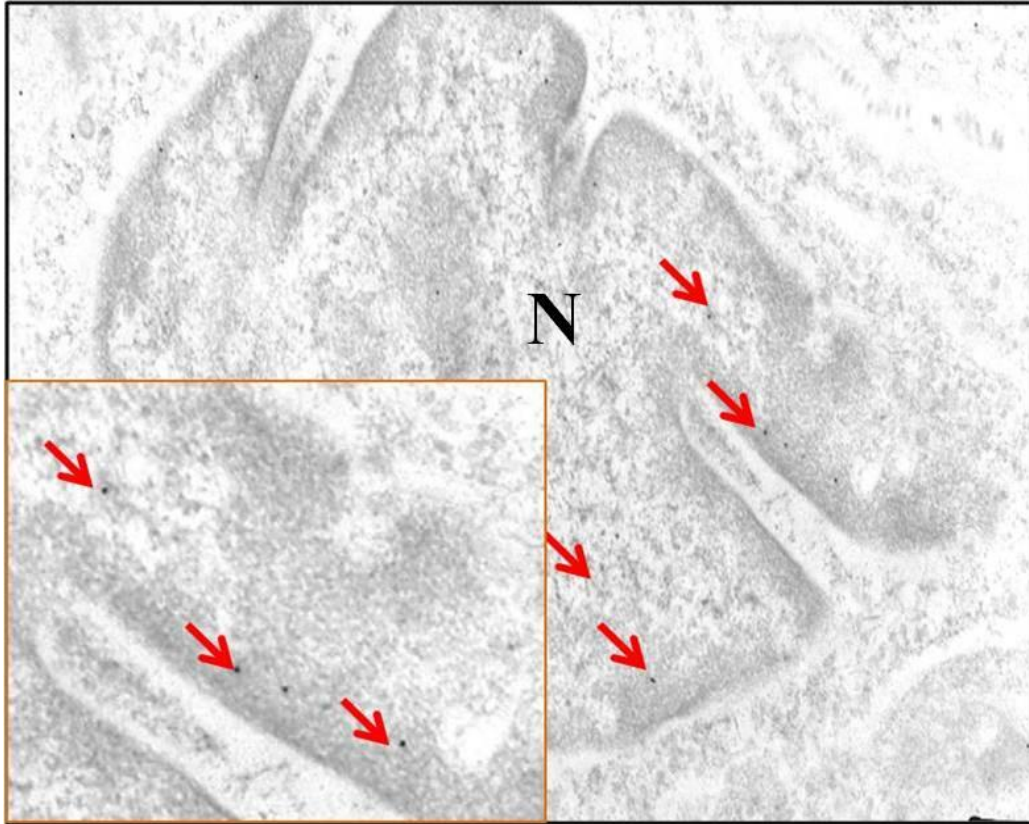


Figure 4.10. Immunoelectron microscopy for retinoid receptors in normal lungs. Arrows show labeling for RAR γ in human neutrophil. N: Nucleus, C: Cytoplasm. Original magnification: 13000X

4.6. Discussion

Retinoids are necessary for normal lung development and function (Zachman 1995; Kassaye, Becklake et al. 2001). Retinoids also play a critical role in optimum function of the immune system (Montrone, Martorelli et al. 2009). Vitamin A deficiency has been shown to cause airway hyperresponsiveness and it is reversed by retinoic acid treatment (McGowan, Holmes et al. 2004). Retinoids affect the functioning of important immune cells such as macrophages and neutrophils. They inhibit cytokine release from macrophages activated by endotoxin and inflammatory cytokines such as TNF- α (Mehta, McQueen et al. 1994). These important physiological effects of retinoids are mediated through retinoid receptors which include retinoic acid receptors (RARs) and retinoid X receptors (RXRs) with α , β , and γ subtypes (Gudas 1994). Based on the previous studies and their role in inflammation, I hypothesised that retinoid receptor expression is altered in acute lung inflammation. Because of the importance of receptors in transduction of cellular effects of retinoids and because of highly limited data on their expression in lung cells, we examined the expression pattern of retinoid receptors in normal and inflamed mouse and human lungs. One of the strengths of this study is the ultrastructural localization of various receptors in the lungs. While immunohistology provides data on the localization of proteins in various cells, the cell-specific subcellular localization such as retinoid receptors in the nucleus is important to understand their functions.

Western blot indicated expression of all retinoid receptor subtypes in normal mouse lungs. Immunohistochemical staining showed expression of retinoid receptors in alveolar/septal macrophages, bronchial epithelial cells, vascular endothelium and alveolar epithelial cells. Among all the retinoid receptor subtypes, RAR γ was widely expressed and its expression was found in all the four cell types of lungs. Airway epithelium showed positive staining for all the

subtypes of retinoid receptors except $RAR\alpha$. Airway epithelium plays an important role in inflammation. It regulates lung inflammation through the NF- κ B pathway (Cheng, Han et al. 2007). Airway epithelial cells also produce several mediators of inflammation and act as effectors of inflammation (Martin, Rochelle et al. 1997). Retinoids are important for maintaining the epithelial integrity, and retinoic acid has been shown to reverse the airway hyperresponsiveness (McGowan, Holmes et al. 2004). The constitutive expression of retinoid receptors in airway epithelium may facilitate maintenance of epithelial barrier under normal and inflamed conditions.

Alveolar/septal macrophages also showed positive staining for $RAR\alpha$, $RAR\gamma$, $RXR\beta$, and $RXR\gamma$. These are the first data on expression of retinoid receptor subtypes in alveolar/septal macrophages. Expression of retinoid receptors in alveolar macrophages may indicate their involvement in lung inflammation because retinoic acid inhibits production of proinflammatory cytokines such as TNF- α and increases production of antiinflammatory cytokines such as interleukin-10 (Wang, Allen et al. 2007). Retinoid receptors signaling plays a critical role in regulating the inflammatory response in macrophages (Valledor and Ricote 2004), and our immuno-electron microscopic data showed clear nuclear localization of retinoid receptors in alveolar macrophages and neutrophils.

Neutrophils, similar to alveolar macrophages are central to pathogenesis of acute lung injury (Martin 2002). Activation of human neutrophils leads to synthesis of $RAR\alpha$ protein which modulates production of IL-8 (Yost, Denis et al. 2004). On the other hand, treatment with retinoic acid inhibits production of reactive oxygen species by neutrophils (Varani, Jones et al. 1991) to suggest their important roles in modulating neutrophil behaviour and influencing acute lung inflammation.

Expression of retinoid receptors was also found in vascular endothelium. Altered vascular permeability is a hallmark of acute lung injury and is an outcome of damaged endothelial cell junctions (Maniatis, Kotanidou et al. 2008; Maniatis and Orfanos 2008). The expression of retinoid receptors may be important in the context of the role of retinoic acid in protection of the barrier properties of vascular endothelium (Osanai, Nishikiori et al. 2007). Retinoic acid decreases TNF- α induced expression of tissue factor in vascular endothelial cells preserving the anti-inflammatory phenotype of normal endothelium (Marchetti, Vignoli et al. 2003). The constitutive endothelial expression of retinoid receptors that can suppress production of reactive oxygen species and inhibit NF-kB may indicate their role in the functioning of endothelial cells. Following further characterization of the specific functions of retinoid receptors, it may be possible to use them as therapeutic targets. In addition to mouse lungs, we also studied expression of retinoid receptors in normal human lungs. There have been previous studies that have reported expression of retinoid receptors in human lungs. These studies were done mainly to determine the expression in fetal lungs and expression was found in epithelial cells and mesenchymal cells (Kimura, Suzuki et al. 2002; Rajatapiti, Kester et al. 2005). However, expression in specific cell types such as alveolar macrophages, vascular endothelium and neutrophils which are important in lung inflammation has not been characterized. Expression pattern of retinoid receptors was different from that of mouse lungs. For example, RAR α was expressed both in alveolar macrophages and vascular endothelium in mouse lungs. However, in human lungs RAR α expression was found only in vascular endothelium. These data indicate distinct expression pattern of retinoid receptors in each species. Differences in expression of retinoid receptor subtypes between mouse and human lungs indicate that there may be differential regulation of gene expression by retinoids in each species.

After characterizing the expression pattern in normal mouse and human lungs, I then determined expression in inflamed lungs. Western blot followed by densitometry indicated that expression levels of RXR α were increased in inflamed mouse lungs compared to normal lungs. I also observed increased intensity of staining for RXR α in the airway epithelium of LPS-treated mouse lungs compared to saline-treated animals. Airway epithelium plays an important role in lung inflammation through production of cytokines and chemokines as well as barrier function (Cheng, Han et al. 2007). Altered airway expression of retinoid receptors has been shown in chronic inflammatory condition such as asthma. Increased expression of retinoid receptors in airway epithelium was correlated with morphologically intact epithelium (Druilhe, Zahm et al. 2008). Retinoic acid has also been found to inhibit interleukin-4-induced eotaxin production in a human bronchial epithelial cell line (Takamura, Nasuhara et al. 2004). In the context of increased expression in acute lung injury the role of RXR α needs further investigation. The increased epithelial expression of RXR α may be part of the protective response and to strengthen the barrier to protect against leakage and neutrophil influx. There were no changes in protein and mRNA levels of other receptor subtypes (data not shown). In inflamed human lungs I saw massive inflammatory cell infiltration and tissue damage. Although I observed inflammatory cells positive for retinoid receptors, number of positive cells was not different from that of control lungs. Since the lung tissues were collected after the death of patients diagnosed with sepsis, the possibility of their altered expression during other stages of sepsis cannot be ruled out. The expression of retinoid receptors is a time-dependent phenomenon (Liebler, Uberschar et al. 2004). Also, the number of human cases needs to be increased to further validate the data. Therefore, analysis of expression at different stages of inflammation is necessary.

Taken together, we have shown expression of retinoid receptors in different cell types in mouse and human lungs. Increased expression of RXR α in the airway epithelium of inflamed mouse lungs partially proved my hypothesis. The increased expression indicates its possible role in airway epithelial function in inflammation. Retinoid receptor expression in alveolar macrophages and vascular endothelium also indicates retinoid receptor signaling in these cell types. Therefore, elucidating the role of retinoid receptor signaling in these cell types will be helpful in understanding the role of retinoids in lung inflammation and may open new therapeutic targets to treat lung inflammation.

CHAPTER 5: EFFECT OF RETINOIC ACID ON CANINE NEUTROPHIL APOPTOSIS

5.1. Abstract

Neutrophils are the key cellular component of the innate immune system. Activated neutrophils live longer, produce toxic metabolites and cause considerable tissue injury, which is central to the pathogenesis of many inflammatory conditions. Retinoids are a class of lipophilic compounds that include natural vitamin A and its synthetic derivatives with anti-inflammatory effects. I examined the effect of retinoic acid on apoptosis in normal and activated neutrophils using light microscopy, electron microscopy, terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay, and Annexin V staining. Our results showed that treatment with 1 $\mu\text{g/ml}$ *E. coli* lipopolysaccharide (LPS) for 12 and 36 hours delayed the spontaneous neutrophil apoptosis compared to untreated cells ($p < 0.05$). But treatment of cells with both LPS (1 $\mu\text{g/ml}$) and retinoic acid (1 and 5 μM) abolished the inhibitory effects of LPS on neutrophil apoptosis in a concentration- dependant manner ($p < 0.05$). Flow cytometry data indicated significant differences in number of apoptotic cells to indicate plasma membrane changes during early stages of apoptosis. TUNEL assay showed nuclear staining indicative of DNA fragmentation, and light microscopic examination indicated nuclear condensation in apoptotic neutrophils. Electron microscopic examination showed induction of nuclear and other cellular changes to indicate apoptosis in neutrophils. However, there were no changes in caspase-3 activity. These results provide new insights on the effects of retinoic acid on neutrophil apoptosis and this effect could enhance the resolution of inflammation.

5.2 Introduction

Neutrophils are the most abundant white blood cells and they play an important role in the innate immune system through phagocytosis and killing of pathogens (Appelberg 2007).

Although neutrophils are critical for host defence, extracellular release of proteases and reactive oxygen species at the site of inflammation leads to considerable tissue damage which has been considered central to the pathogenesis of inflammatory diseases (Holman and Saba 1988; Smith 1994; Appelberg 2007). Neutrophils play a key role in the pathogenesis of acute lung injury (Rinaldo 1986; Grommes and Soehnlein 2011) and severity of lung injury is reduced following depletion of neutrophils (Folz, Abushamaa et al. 1999). Neutrophils are terminally differentiated cells and programmed to undergo spontaneous apoptosis. Short life span and spontaneous apoptosis of neutrophils is due to constitutive expression of pro-apoptotic proteins and lack of anti-apoptotic proteins (Akgul, Moulding et al. 2001). However, apoptosis of neutrophils is delayed at the site of inflammation by a number of pathogen and host derived inflammatory mediators (Lee, Whyte et al. 1993; DeLeo 2004). This leads to accumulation and prolonged activation of neutrophils which further aggravates neutrophil-induced tissue injury (Mecklenburgh, Murray et al. 1999). Neutrophil apoptosis is delayed in acute lung injury (Lin, Lin et al. 2011). The inflammatory mediators activate pathways such as phosphatidylinositol 3-kinase (PI3-K), mitogen activated protein kinase/extracellular signal regulated kinases (MAPK/ERK), and p38 mitogen activated protein kinase (p38MAPK) to delay the spontaneous apoptosis of neutrophils (Brizzi, Aronica et al. 1996). Furthermore, apoptotic neutrophils protect from lipopolysaccharide-induced sepsis (Ren, Xie et al. 2008).

Retinoids are a group of compounds that include vitamin A, its biological and synthetic derivatives. Retinoids possess anti-inflammatory properties through their inhibitory effects on transcription factors such as NF- κ B and activator protein-1 (Kuenzli, Tran et al. 2004). Retinoic acid is the active metabolite and biologically most active retinoid (Dolle, Ruberte et al. 1990). It has a profound influence on neutrophil biology. Retinoic acid binds to retinoid receptors within

the nucleus to regulate gene expression (Evans 1988). Although retinoic acid binds to retinoic acid receptors (RARs), they regulate gene expression by forming heterodimers with retinoid X receptors (RXRs) (Kliwer, Umesono et al. 1992). Retinoic acid promotes differentiation of immature neutrophils to mature neutrophils (Huang, Ye et al. 1988). Deficiency of vitamin A in mice results in the abnormal expansion of myeloid cells with impaired apoptosis in neutrophils (Kuwata, Wang et al. 2000). In addition, vitamin A deficiency has been shown to exacerbate the inflammatory response with neutrophilia (Wiedermann, Chen et al. 1996). Retinoids also affect apoptosis of immune cells. Retinoic acid has been shown to exert pro-apoptotic effects on T-lymphocytes and anti-apoptotic effects on eosinophils (Szondy, Reichert et al. 1998; Ueki, Mahemuti et al. 2008). However, there are no data on the effect of retinoic acid on apoptosis of either resting or activated neutrophils.

Since the retinoids have been shown to possess anti-inflammatory properties and impaired apoptosis has been reported in vitamin A deficient animals and in activated neutrophils, we investigated the effects of retinoic acid on neutrophil apoptosis *in vitro*. Therefore, the objective of this study was to determine the effect of retinoic acid on spontaneous apoptosis of resting and activated canine neutrophils.

5.3. Materials and Methods

5.3.1. Reagents

Retinoic acid, lipopolysaccharide (*Escherichia coli* O11:B4), and camptothecin were purchased from Sigma Aldrich (St. Louis, MO). Annexin V and Propidium iodide (PI) kit was purchased from BD biosciences (Mississauga, Canada). *In situ* cell death detection kit was purchased from Roche Applied Sciences (Laval, Canada). May-Grunwald and Giemsa stain were

purchased from VWR International. Caspase-3 colorimetric kit was purchased from Millipore (Billerica, USA).

5.3.2. Blood collection and neutrophil isolation

5.3.2.1. Blood collection: Blood samples were collected from clinically healthy dogs and diluted by mixing 1.5 ml of blood with 0.5 ml of normal saline just before the isolation.

5.3.2. 2. Neutrophil isolation

Neutrophils were isolated as per the previously described method with few modifications (Comazzi, Paltrinieri et al. 2001). Briefly, equal volumes of isotonic percoll solutions of different densities (1.096 and 1.072) were overlaid on each other. Blood was carefully layered over percoll gradients and centrifuged at 400 g for 20 min. A band of PMNs formed between two percoll gradients was collected and contaminating erythrocytes were lysed by adding 5 ml of sterile distilled water for 1 min and isotonicity restored by adding 2.5 ml of 0.3 mol/L NaCl in 0.0132 mol/l phosphate buffer pH 7.2. The cell suspension was centrifuged at 400 g for 12 min and washed twice with phosphate buffer saline. Neutrophils were resuspended in 1 ml RPMI 1640 media and counted to determine the cell number and the cell viability was assessed by trypan blue exclusion. The cell suspension was cytocentrifuged, stained with May-Grunwald Giemsa and differential cell count performed to determine the purity of cells. The purity of neutrophils was always >95%.

5.3.3. Cell treatments

Neutrophils were suspended (2×10^6 cells per ml) in RPMI 1640 (Sigma Aldrich, St. Louis, MO) supplemented with 5% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). The cells were treated with media alone or with LPS (1 µg/ml). To determine the effects of retinoic acid, some groups were incubated with both LPS and retinoic acid (1 and 5

μM). Sham controls of only retinoic acid (1 and 5 μM), a vehicle group (Dimethyl sulfoxide), and as positive control, camptothecin (10 μM) were also used. Cells were incubated with these reagents at 37 °C in a humidified 5 % CO₂ incubator for 12 and 36 hours. Flow cytometry was performed on neutrophils incubated for 8 hours.

5.3.4. Flow cytometry

For flow cytometry, isolated cells were incubated for 8 hours with either LPS (1 μg/ml) alone or LPS plus retinoic acid (1 and 5 μM), and the assay was done using FITC Annexin V Apoptosis Detection Kit (BD biosciences, Mississauga, Canada) as per manufacturer's instructions. Briefly, after the treatment period, cells were washed twice with PBS and suspended in annexin V binding buffer (0.1 M Hepes/NaOH, 1.4 M NaCl; 25 mM CaCl₂). Cells were stained with propidium iodide and FITC-annexin V for 15 minutes at room temperature and analyzed by flow cytometry within 1 h.

5.3.5. Caspase-3 assay

Caspase-3 assay was done using caspase-3 colorimetric activity assay kit (Millipore Corporation) as per the manufacturer's instructions. Briefly, after the treatment, neutrophils were washed twice with PBS and suspended in lysis buffer to extract the cellular proteins. Cell lysates were incubated with DEVD-pNA substrate for 1 h at 37°C. After the incubation the free pNA was quantified by measuring absorbance at 405 nm in a microtiter plate reader. Recombinant human caspase-3 was used to plot the standard curve and calculate caspase-3 units.

5.3.6. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL Assay)

TUNEL assay was done using *In Situ* Cell Death Detection Kit (Roche Applied Science, Laval, Quebec, Canada) as per the manufacture's instruction. Briefly, cytopsin slides were fixed with 4% paraformaldehyde, and cells were permeabilized with 0.1% Triton X in 0.1% sodium citrate for 2 minutes at 4° C. Afterwards, the slides were incubated with TUNEL reaction

mixture for 1 h at 37° C in a humidified chamber. The slides were washed thrice and incubated with converter- AP for 30 min at 37° C in a humidified chamber. Finally, the slides were incubated with substrate solution (NBT/BCIP) for 10 minutes at room temperature in the dark. The slides were mounted with PBS/glycerol, and 500 cells were counted and differentiated as positive and negative cells based on nuclear staining. Cell numbers were converted to percent apoptotic cells for statistical analysis.

5.3.7. Light microscopy

After the indicated treatments, cells were pelleted, washed twice with PBS and cytocentrifuged. The cytospin slides were stained with May-Grunwald Giemsa stain and 500 cells were counted in a blinded fashion, differentiating cells as apoptotic or normal cells based on nuclear morphology. Cell numbers were converted to percent apoptotic cells for statistical analysis.

5.3.8. Electron microscopy

After the treatment period cells were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer for 3 hours at 4°C and then post fixed in 0.1 % osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h at room temperature. The cells were dehydrated with a graded series of alcohol embedded in Epon/Araldite and polymerized overnight at 60°C. Cells were differentiated as normal or apoptotic based on nuclear morphology.

5.3.9. Statistical Analysis

The data obtained from three independent experiments were expressed as mean \pm standard error. Statistical analysis was performed by one-way ANOVA with Tukey's post hoc test (GraphPad Prism Software, San Diego, CA). A 'p' value of ≤ 0.05 was considered as statistically significant.

5.4. Results

5.4.1. Flow cytometry

We used double staining with annexin V and propidium iodide to determine the effect of retinoic acid on neutrophil apoptosis. Translocation of phosphatidylserine to the extracellular surface of the cell membrane is one of the earliest changes during apoptosis and FITC-tagged annexin V is used to label phosphatidylserine (Andree, Reutelingsperger et al. 1990; Fadok, Voelker et al. 1992). Propidium iodide permeabilizes into cell through a compromised cell membrane in necrotic cells but not in apoptotic cells (Vitale, Zamai et al. 1993). As shown in Figure 5.1, quadrant III (Q3) represents annexin V positive cells or apoptotic cells. In untreated cells, apoptosis was significantly more compared to LPS treated cells (Figure 5.2). However, both concentrations of retinoic acid (1 and 5 μ M) significantly increased neutrophil apoptosis in the presence of LPS (Figure 5.2).

5.4.2. Caspase-3 assay

Caspase-3 is a protease that exists as an inactive proenzyme in normal cells. However, it is activated to mediate a number of changes during apoptosis (Alnemri, Livingston et al. 1996). It cleaves protein molecules with amino acid motif DEVD and this principle is employed in colorimetric assay for caspase-3 (Casciola-Rosen, Nicholson et al. 1996). Incubation of canine neutrophils with different reagents in our experiments did not produce significant differences between groups for the caspase-3 activity (Figure 5.3).

5.4.3. TUNEL Assay

DNA fragmentation is a characteristic event in terminal stages of apoptosis and DNA fragments are labelled by fluorescent tagged nucleotides using terminal transferase enzyme. This technique is called as Terminal dUTP Nick End-Labeling (Gavrieli, Sherman et al. 1992).

Apoptotic neutrophils were identified as TUNEL positive while the normal neutrophils lacked nuclear staining (Figure 5.4A-D). Incubation of neutrophils with LPS for 12 and 36 hours resulted in significant decrease in apoptosis compared to untreated cells (Figure 5.5). However, in the presence of retinoic acid (1 and 5 μ M) the effect of LPS on apoptosis was inhibited as shown by a significant increase in neutrophil apoptosis compared to LPS alone. After 12 hours of incubation both the concentrations of retinoic acid significantly reversed the effect of LPS. However, after 36 hours only higher concentration (5 μ M) induced significant increase in apoptosis. Interestingly, treatment with only retinoic acid resulted in significant inhibition of neutrophil apoptosis compared to untreated cells (Figure 5.5)

5.4.4. Light microscopy

Light microscopy detects apoptosis based on the nuclear morphology. Normal healthy neutrophils were identified by their characteristic multilobed nucleus (Figure 5.6A-D). In contrast, apoptotic neutrophils were identified by condensed nucleus and nuclear bodies. Treatment of canine neutrophils with LPS (1 μ g/ml) resulted in a significant reduction in the number of apoptotic cells compared to untreated cells at both 12 and 36 hours (Figure 5.7). However, incubation with both retinoic acid (1 and 5 μ M) and LPS resulted in significant and dose dependent increase in apoptotic cells after 12 hours (Figure 5.7). After 36 hours, only higher concentration of retinoic acid produced significant effect. Similar to TUNEL assay light microscopy results also indicated significant inhibition of neutrophil apoptosis when treated with only retinoic acid compared to untreated group.

5.4.5. Electron microscopy

Electron microscopy for apoptosis is based on characteristic ultrastructural morphological changes in apoptotic cells and was used to confirm apoptosis in the cells (Wyllie, Kerr et al.

1980). Neutrophils exposed to LPS showed characteristic multilobed nucleus, ruffled plasma membrane and granules of various densities (Figure 5.8-A). In contrast, neutrophils treated with both LPS and RA showed signs of apoptosis such as nuclear condensation, apoptotic bodies in the cytoplasm and shrunken size (Figure 5.8-B).

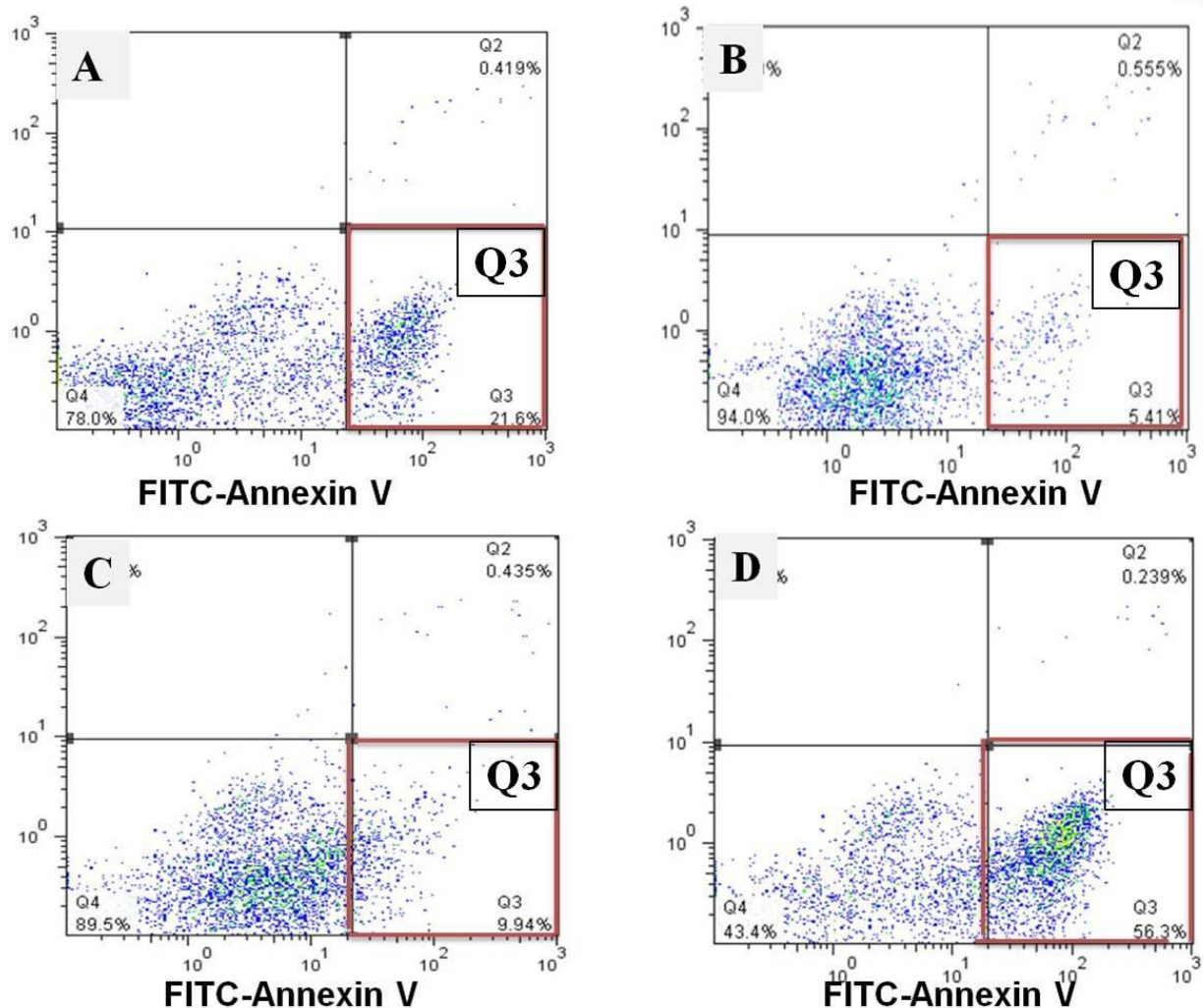


Figure 5.1: Representative flow cytometric data of neutrophil apoptosis. Neutrophils were incubated with medium alone (A), with LPS alone (1 μ g/ml; B), with LPS plus retinoic acid (5 μ M; C), and camptothecin (10 μ M; D) for 8 hours. After the treatment period, cells were stained with FITC-labeled annexin V and PI the fluorescence was determined by flow cytometry. The results are representative of three independent experiments. Q3 represents annexin-V positive cells/apoptotic cells.

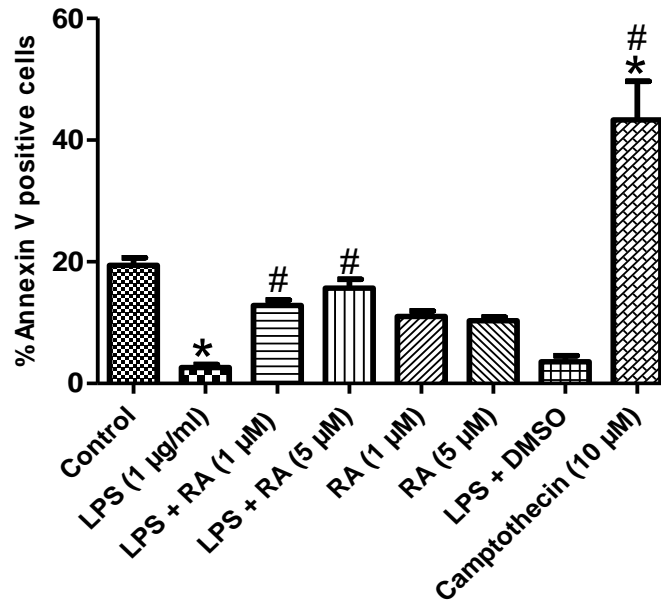


Figure 5.2: Flow cytometric analysis of canine neutrophil apoptosis. Canine neutrophils were incubated without any treatment (Control), with either LPS (1µg/ml) alone, or LPS plus retinoic acid (1 & 5 µM) for 8 hours. After treatment period Annexin V and propidium iodide double staining was done. Data represent mean \pm SE of three independent experiments. * $P < 0.05$ versus control, # $P < 0.05$ versus LPS.

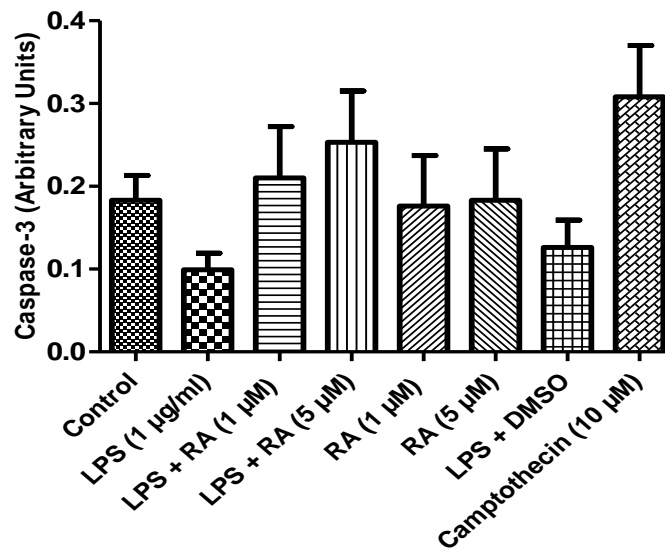


Figure 5.3: Caspase-3 assay in canine neutrophils. Canine neutrophils were incubated without any treatment, with LPS (1µg/ml) alone, or LPS plus retinoic acid (1 & 5 µM) for 12 hours. After the treatment period, caspase-3 assay was done. Data represent mean \pm SE of three independent experiments.

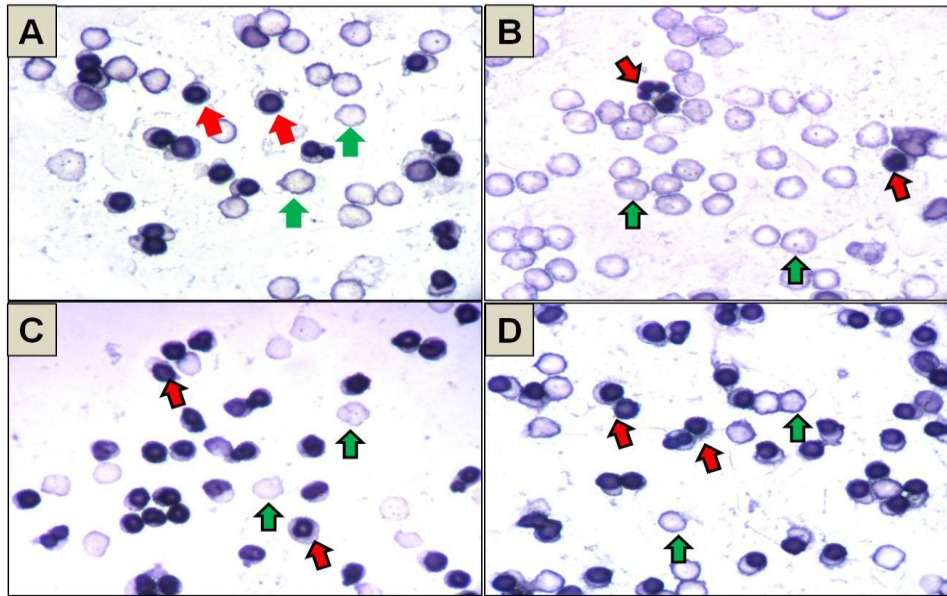


Figure 5.4: Representative pictures of TUNEL assay. Neutrophils were incubated with no treatment (A), with LPS alone (1 µg/ml; B), with LPS (1µg/ml) plus retinoic acid (5 µM; C), and camptothecin (10 µM; D) for 12 hours. After incubation, TUNEL assay was done on cytopins, and examined by light microscopy. Healthy neutrophils with no nuclear staining are indicated by green arrows and apoptotic neutrophils with nuclear staining are indicated by red arrows. Representative pictures of three independent experiments.

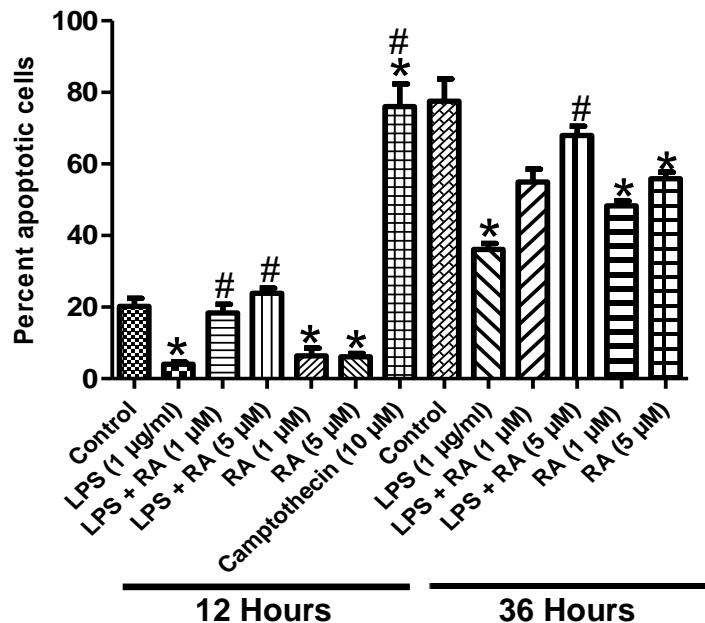


Figure 5.5: TUNEL assay of neutrophil apoptosis. Canine neutrophils were incubated without any treatment, with either LPS (1µg/ml) alone, or LPS plus retinoic acid 1 & 5 µM) for 12 and 36 hours. After treatment periods, TUNEL assay was done and 500 cells were counted from each treatment group and expressed as percent apoptotic cells. Data represent mean ± SE of three independent experiments. * P < 0.05 versus control, # P < 0.05 versus LPS.

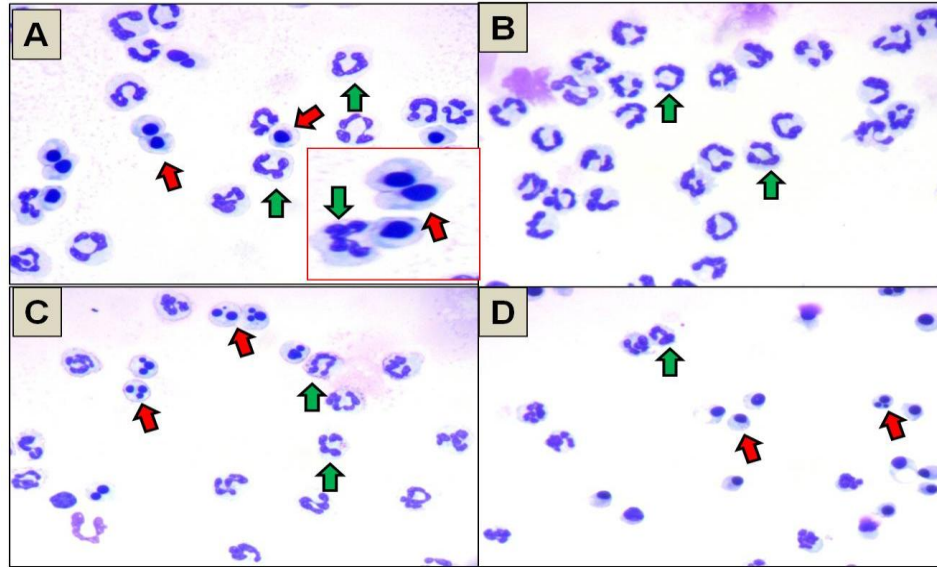


Figure 5.6: Representative pictures of light microscopy. Neutrophils were incubated with no treatment (A), with LPS alone (1 $\mu\text{g/ml}$; B), with LPS (1 $\mu\text{g/ml}$) plus retinoic acid (5 μM ; C), and camptothecin (10 μM ; D) for 12 hours. After incubation, cytopins were prepared and stained for light microscopy. Healthy neutrophils (green arrows) with characteristic multilobed nucleus and apoptotic neutrophils (red arrows) show condensed nucleus. Representative pictures of three independent experiments.

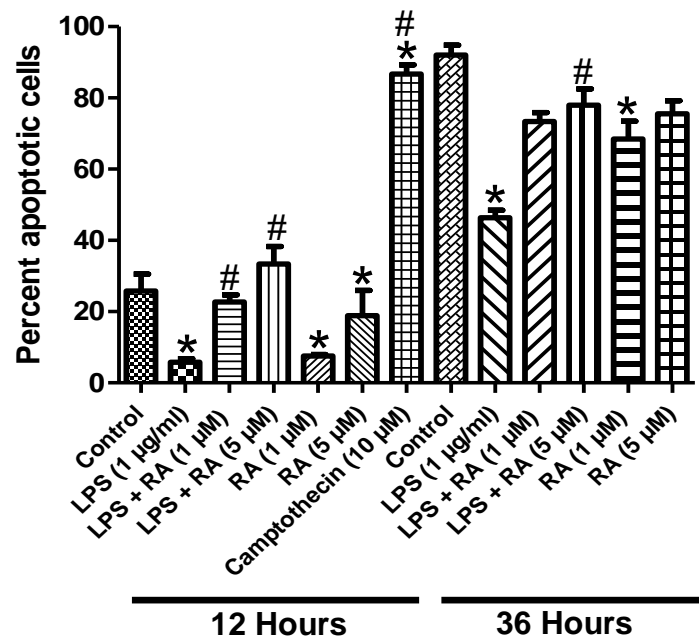


Figure 5.7: Light microscopic analysis of neutrophil apoptosis. Neutrophils were incubated with no treatment (A), with LPS alone (1 $\mu\text{g/ml}$; B), with LPS (1 $\mu\text{g/ml}$) plus retinoic acid (5 μM ; C), and camptothecin (10 μM ; D) for 12 hours and 36 hours. After incubation, cytopins were stained for light microscopy, 500 cells were counted from each group and cell numbers were converted to percent apoptotic cells. Data represent mean \pm SE of three independent experiments. * $P < 0.05$ versus control, # $P < 0.05$ versus LPS.

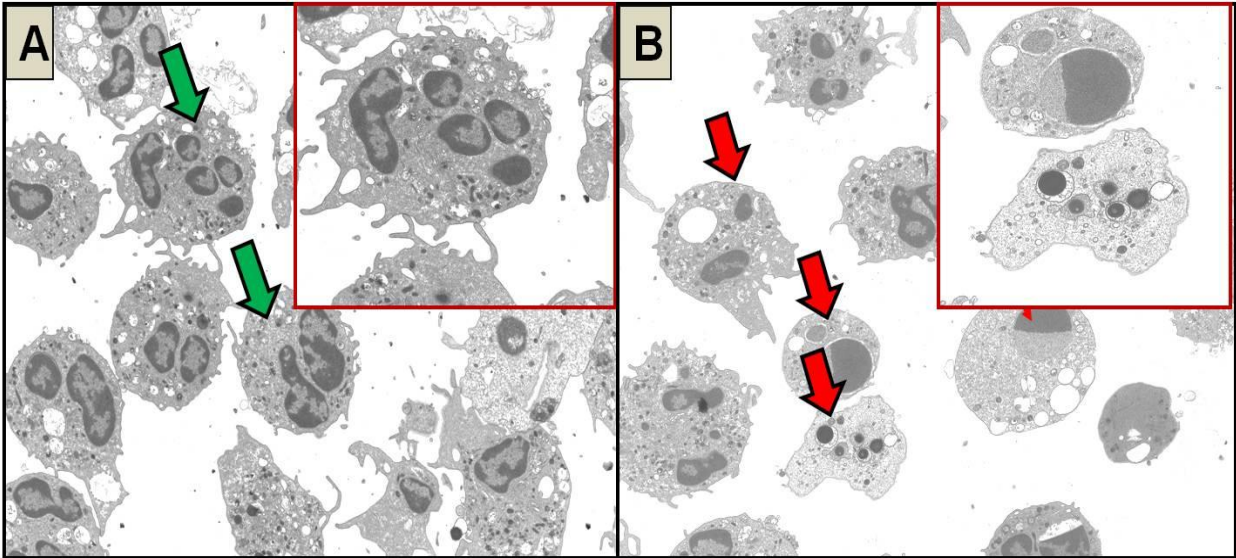


Figure 5.8: Representative pictures of electron microscopic analysis of neutrophil apoptosis. Neutrophils were incubated with LPS alone (1 µg/ml; A), with LPS (1 µg/ml) plus retinoic acid (5 µM; B) for 12 hours. After incubation, electron microscopy was done for morphological analysis. Normal neutrophils (green arrows) show characteristic multilobed nucleus where as apoptotic neutrophils (red arrows) show chromatin condensation and rounded nuclear bodies

5.5. Discussion

Neutrophils are essential component of the innate immune system and play an important role in first line defence against invading pathogens. However, accumulation of activated neutrophils at the site of injury or infection causes considerable tissue damage through secretion of reactive oxygen intermediates, proteolytic enzymes and cytokines (Segel, Halterman et al. 2011). Neutrophil-induced tissue injury has been found to play a key role in several inflammatory diseases including acute lung injury (Fujishima and Aikawa 1995; Witko-Sarsat, Rieu et al. 2000). Neutrophils recruited to the site of inflammation undergo spontaneous apoptosis and are cleared by macrophages through phagocytosis. This is important in resolution of inflammation and reduction of tissue injury (Savill, Wyllie et al. 1989; Chilvers, Cadwallader et al. 2000). Microbial and host derived products activate neutrophils and prolong their lifespan by delaying their constitutive apoptosis (Lee, Whyte et al. 1993). Activated neutrophils with prolonged lifespan are believed to cause significant tissue damage through production of toxic molecules. One of the ways to modulate the behaviour of activated neutrophils is to find ways to regulate their lifespan.

Retinoids are known for their anti-inflammatory effects and they also affect neutrophil biology (Lawson and Berliner 1999). Retinoids also exerts apoptosis on various cell types and impaired neutrophil apoptosis has been reported in vitamin A deficient animals (Kuwata, Wang et al. 2000; Noy 2010). Based on these data I hypothesised that retinoic acid induces apoptosis of canine neutrophils. Here I provide the data that retinoic acid can reverse the life prolonging effects of LPS on canine neutrophils. My data show that bacterial LPS delays spontaneous apoptosis of neutrophils and simultaneous treatment with retinoic acid abolishes this effect. Previous studies have shown that LPS-induced delay in neutrophil apoptosis is mediated through

activation of MAPK and ERK pathways (Akgul, Moulding et al. 2001; Riedemann, Guo et al. 2004). Therefore, it is possible that retinoic acid may inhibit these pathways in LPS-activated neutrophils to promote apoptosis. Retinoic-induced apoptosis in various cell lines is mediated both through intrinsic and extrinsic pathways. Retinoic acid induces apoptosis through upregulation of pro-apoptotic factors such as Bcl-2 proteins, transcription factors and genes involved in DNA fragmentation. Pro-apoptotic effects of retinoic acid are mediated by upregulation of pro-apoptotic Bcl-2 proteins such as Bax (Niu, Menard et al. 2001). Retinoic acid induces apoptosis through downregulation of antiapoptotic Bcl-2 proteins and survivin (Raffo, Emionite et al. 2000; Pratt, Niu et al. 2003). Retinoic acid also induces upregulation of tumor suppressor p53 to exert pro-apoptotic effects (Thin, Li et al. 2007). Pro-apoptotic effects of retinoic acid through extrinsic pathway are mediated by upregulation of TNF-related apoptosis-inducing ligand leading to the activation of death receptors (Altucci, Rossin et al. 2001). Activation of death receptor Fas also mediates retinoic acid induced apoptotic effects (Engedal, Auberger et al. 2009). Retinoic acid induces expression of receptors for TNF- α and promotes TNF- α induced apoptosis (Manna and Aggarwal 2000; Witcher, Ross et al. 2003). The exact pathway of retinoic acid-induced apoptosis in activated canine neutrophils needs to be elucidated.

These results also indicate that retinoic acid might exert its anti-inflammatory effects by accelerating neutrophil apoptosis and reversing the effects of inflammatory mediators at the site of inflammation. These effects of retinoic acid on apoptosis of neutrophils might prevent accumulation of activated neutrophils at the site of inflammation resulting in reduced tissue damage and promotion of resolution. Studies have shown exaggerated inflammatory response in vitamin A deficiency (Wiedermann, Chen et al. 1996). This could be due to impaired apoptosis

of activated neutrophils coupled with activation of transcription factors such as NF- κ B. Retinoic acid inhibits LPS-induced activation of NF- κ B through induction of new protein called signal transducer and activator of transcription-1 (Austena, Carlsen et al. 2009). Retinoic acid also inhibits NF- κ B-DNA interaction to suppress cytokine production by LPS-activated macrophages (Na, Kang et al. 1999). Further studies are necessary to determine *in vivo* implications of pro-apoptotic effects of retinoic acid.

I explored mechanisms of pro-apoptotic effects of retinoic acid on activated neutrophils through examination of caspase-3 expression. Caspase-3 plays a central role as an executioner enzyme in apoptosis (Porter and Janicke 1999; Zimmermann, Bonzon et al. 2001). Interestingly, I did not find significant differences in caspase-3 activity between treatment groups after 12 hours treatment. This is not surprising since apoptosis is a complex process with different changes occurring at different time points (Saraste and Pulkki 2000). It is possible that I may have missed the time point of differences in caspase-3 expression as I examined only two time points. Further studies are needed to explore the mechanism of retinoid acid-induced apoptosis of activated canine neutrophils. Interestingly, present study also showed inhibition of apoptosis in resting neutrophils by retinoic acid. Previous studies have shown that retinoic acid treatment induces anti-apoptotic pathways following short term treatment and I presume that this could result in inhibition of neutrophil apoptosis (Liu, Zhang et al. 2000). It also indicates that retinoic acid exerts differential effects on resting and activated neutrophils, which shows complexity of retinoic acid signaling. Notwithstanding the lack of precise mechanistic evidence, the multiple lines of evidence, one of the strengths of this study, clearly show that retinoic acid induces apoptosis in activated neutrophils.

Retinoic acid has been shown to suppress transcription factors such as NF- κ B and activator protein-1 and these effects have been attributed to anti-inflammatory mechanism of retinoic acid. My results show that retinoic acid abolishes the effects of LPS on spontaneous apoptosis, and it might be reasonable to presume that the antiinflammatory effects of retinoic acid might also involve acceleration of neutrophil apoptosis, in addition to previously proposed mechanisms. Retinoids are known for their anti-inflammatory properties and supplementation of vitamin A has been shown to suppress inflammatory response (Reifen, Nur et al. 2002; Torii, Miyake et al. 2004). Furthermore, the data from our experiment indicate that retinoic acid accelerate apoptosis in activated neutrophils. The results indicate a new mechanism of anti-inflammatory effects of retinoic acid. Since there was no pro-apoptotic effect on resting canine neutrophils, my hypothesis is partially proved. Further studies are required to elucidate the molecular mechanisms of retinoic acid-induced apoptosis of activated neutrophils, and effects of retinoic acid on the production of reactive oxygen species and production of inflammatory mediators by activated canine neutrophils.

CHAPTER 6: EFFECTS OF RETINOIC ACID ON ENDOTOXIN-INDUCED ACUTE LUNG INFLAMMATION

6.1. Abstract

The pathogenesis of acute lung injury is attributed to inflammatory damage induced mainly by neutrophils. Alveolar macrophages and endothelial cells through expression of chemokines facilitate and regulate neutrophil recruitment. Acute lung injury is characterized by compromised endothelial barrier and infiltration of leukocytes into lungs resulting in extensive damage to lung parenchyma. Previous *in vitro* and *in vivo* studies show that the retinoids exert antiinflammatory effects. This study was designed to determine the effect of retinoic acid on acute lung inflammation using endotoxin-induced murine acute lung injury model. Effect of retinoic acid on canine neutrophil chemotaxis was also studied *in vitro*. Animals were pre-treated with retinoic acid (10 mg/kg, intraperitoneally), 30 minutes before intranasal instillation of 80 µg *E. coli* lipopolysaccharide (LPS). Lung inflammation was assessed at 6, 12, and 24 hours after LPS treatment. Retinoic acid pre-treatment significantly reduced the number of total cells, percentage of neutrophils and protein concentration in bronchoalveolar lavage fluid at 6 hours following LPS treatment ($p < 0.05$). Retinoic acid pre-treatment also significantly decreased the numbers of myeloperoxidase-positive cells in lung tissues of LPS-treated mice. However, there was no effect of retinoic acid on TNF- α , IL-1 β , IL-10 and KC levels in bronchoalveolar lavage fluid. There was no effect of retinoic acid on canine neutrophil chemotaxis. Based on *in vivo* data, retinoic acid partially attenuates endotoxin-induced acute lung injury.

6.2. Introduction:

Acute lung injury is a severe clinical condition characterized by pulmonary edema due to an increased vascular permeability, damage to lung parenchyma and accumulation of activated neutrophils. Despite the considerable progress in understanding pathogenesis of acute lung injury

and new therapeutic strategies, the mortality rate in acute lung injury has been reported to be up to 40% (Ware and Matthay 2000). Neutrophils play a pivotal role in pathogenesis of acute lung injury (Abraham 2003). Accumulation of activated neutrophils and fluid in lung alveoli is considered as a histological hallmark of acute lung injury.

Neutrophils are recruited to inflamed lungs through a series of coordinated steps leading to their engagement with vascular endothelium (Reutershan and Ley 2004). The recruitment of neutrophils is facilitated through the expression of chemokines and adhesion molecules on endothelial cells. Activated neutrophils produce anti-microbial molecules such as reactive oxygen species and also eliminate bacteria through phagocytosis (Nauseef 2007). Activated neutrophils have a prolonged lifespan due to their compromised apoptosis. The activated and longer living neutrophils in inflamed lungs lead to considerable tissue damage due to actions of reactive oxygen species and proteolytic enzymes. Such damage to inflamed lungs is believed to induce morbidity and mortality in such patients (Lee and Downey 2001). Therefore, one of the critical needs is to develop mechanisms to preserve beneficial actions of neutrophils while minimizing the tissue damage caused by exuberant activation of neutrophils.

Retinoids are a group of compounds that include vitamin A and structurally related derivatives. *All-trans* retinoic acid is the active metabolite of vitamin A and biologically the most active among all retinoids. The effects of retinoids are mediated through two families of nuclear receptors namely, retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Each family is comprised of three subtypes; α , β and γ (De Luca 1991; Chambon 1996). The heterodimers between a RAR and a RXR subtype is the functional unit that mediates the effects of retinoids. Retinoids possess anti-inflammatory properties through inhibition of transcription factors such as nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1) (Pfahl 1993; Minucci and Ozato

1996). Both these transcription factors mediate production of chemokines and cytokines in response to a variety of inflammatory stimuli (Rahman and MacNee 1998; Tak and Firestein 2001). Vitamin A treatment has been shown to inhibit DNA binding activity of NF- κ B in lungs of ozone exposed rats (Hisada, Adcock et al. 1999). In addition to inhibitory effects on transcription factors, retinoids also inhibit the function of macrophages and neutrophils that play a central role in the pathogenesis of acute lung injury. Retinoic acid inhibits production of reactive oxygen species and proteolytic enzymes from activated human neutrophils. Retinoic acid-treated neutrophils induced less endothelial injury compared to untreated cells (Varani, Jones et al. 1991). *All- trans* retinoic acid has been shown to inhibit inflammatory cytokine production by macrophages activated by lipopolysaccharide (Mathew and Sharma 2000). They also inhibit activation of monocytes by tumor necrosis factor- α and lipopolysaccharide through their inhibitory effect on NF- κ B (Chen et al., 2002). Activation of retinoid receptors by retinoic acid leads to suppression of inflammatory mediators such as metalloproteinases, collagenase, and stromelysin-1 (Fisher, Datta et al. 1996). Taken together, the data published so far show retinoic acid to be an effective anti-inflammatory molecule.

Acute lung injury remains a major cause of mortality and morbidity in humans and animals. The cost of human medical care and the economic impact of lung diseases on the animal industry runs into billions of dollars (Morsey, Van-Kessel et al. 1999; Lush and Kvietys 2000; Rubenfeld and Herridge 2007). Although retinoids have established anti-inflammatory effects, their functions in acute lung inflammation have not been investigated. Considering that there have not been any major breakthroughs in prevention and treatment of acute lung injury, I investigated the effect of pre-treatment with retinoic acid in a murine model of endotoxin-induced acute lung inflammation. In addition, the effect of retinoic acid on chemotaxis of canine neutrophils was also investigated.

6.3. Materials and methods:

6.3.1. Reagents

Lipopolysaccharide (*E. coli*, 055:B5), retinoic acid, and Hank's balanced salt solution were purchased from Sigma Aldrich (Saint Louis, MD). Bio-Plex multiplex ELISA assay kits were purchased from Bio-Rad (Mississauga ON, Canada). Recombinant human interleukin-8 was purchased from PeproTech. Neutrophil chemotaxis chamber and accessories were purchased from Neuro Probe, Inc. (Gaithersburg, USA). Unless otherwise mentioned, all other reagents were obtained from Sigma Aldrich (Saint Louis, MD).

6.3.2. Animals

The experimental protocols were approved by the University of Saskatchewan Committee on Animal Care Assurance and all experiments conducted in accordance with guidelines of the Canadian Council on Animal Care. The male C57BL/6 mice (20–25 g, 8 weeks old) were obtained from Animal Resource Center, University of Saskatchewan. All animals were housed with 12-h dark/light cycle, fed standard laboratory diet and provided ad libitum water.

6.3.3. Experimental design for mouse experiment (Table 1)

Three time points (6 h, 12 h, and 24 h) were used to determine lung inflammation. For each time point there were five groups (n=5/group) of animals. Details on groups and treatment are given in Table 1. Briefly, the first group of mice was treated with endotoxin free saline (80 µl) intranasally. The second group was instilled with LPS (*E.coli* 055:B5; 80 µg in 80 µl saline) by intranasal route. The third group was pre-treated with retinoic acid (10 mg/kg, intraperitoneally) 30 minutes before LPS instillation. The fourth group was treated only with retinoic acid. The fifth group of mice was used as sham-treated group or vehicle group and pretreated with vehicle (Dimethyl sulfoxide) before LPS instillation. At the indicated time points following LPS treatment bronchoalveolar lavage was

done and animals were euthanized to collect lung tissue for further analysis. Bronchoalveolar lavage (BAL) was centrifuged and the supernatant was stored at -80°C for till further analysis. The cell pellet was suspended in Hank's balanced salt solution and cells were counted by hemocytometer.

Table 1. Experimental design

Group	Treatment	Route	Dose
I	Saline	Intranasal	80 µl
II	LPS	Intranasal	80 µg in 80 µl saline
III	Retinoic acid plus LPS	Retinoic acid – I/P	RA – 10 mg/kg
		LPS – Intranasal	LPS – 80 µg
IV	Retinoic acid	Intraperitoneal	10 mg/kg
V	Vehicle (DMSO) plus LPS	Intraperitoneal	1 µl/g

6.3.4. Total and differential cell counts in bronchoalveolar lavage

Total cell count in BAL fluid was done using hemocytometer. The cytopspins were prepared from BALF cell suspension and stained with Hemacolor (EMD Chemicals). Two hundred cells were counted under 400 X magnification with classification as neutrophils, macrophages and expressed as the percentage of total cells.

6.3.5. Immunohistochemistry for lung myeloperoxidase (MPO)

For immunohistochemistry, 5 µm lung sections were cut from paraffin-embedded tissue blocks and placed on polylysine-coated slides. Sections were de-paraffinized, rehydrated and treated with 0.05% hydrogen peroxide to neutralize tissue peroxidase activity. The antigen was

retrieved by incubating sections with pepsin (2 mg/ml in 0.01 N HCl) for 60 min at room temperature and incubated with 1% bovine serum albumin to block non-specific binding. This is followed by incubation with mouse monoclonal antibody (40 µg/ml) to MPO (R & D systems) for 60 min at room temperature. The slides were washed thrice with phosphate buffered saline and incubated with goat antimouse antibody (Dako Canada, Inc, Burlington) for 30 min at room temperature. Color development was done using peroxidase substrate kit (Vector laboratories Inc, Burlington, Canada) and slides were counterstained with methyl green. vWF was used as positive control and primary antibody was omitted for negative controls. MPO positive cells were counted in five randomly selected fields under 400 X magnification.

6.3.6. Protein concentration in bronchoalveolar lavage (BAL) fluid

Protein concentrations in BAL supernatant were measured using Bio-Rad protein assay kit based on the method of Bradford (Bradford 1976). Briefly, in 96-well plates, 10 µl protein samples were mixed with 200 µl dye reagent, incubated for 5 min at room temperature and absorbance was measured at 595 nm. Bovine serum albumin was used to generate the standard curve and used to calculate protein levels.

6.3.7. Cytokine analysis in bronchoalveolar lavage (BAL) fluid

Cytokine levels in BAL supernatant were assayed using Bioplex cytokine assay kits from Bio-Rad laboratories according to manufacturer's instructions. Briefly, in 96-well microtiter plates 50 µl of anti-cytokine beads in assay diluent was added to each well. The plates were washed with Bio-Plex washing buffer and 50-µl volume of sample or standard was added per well. Plates were incubated at room temperature for 30 min in dark on a shaker. A total of 25 µl of biotinylated secondary antibodies was added to each well and plates were incubated at room temperature for 30 min in the dark on a shaker. A 50 µl volume of streptavidin-PE was added to each well, and plates

were incubated at room temperature while being shaken in the dark for 10 min. Samples were read using a Bio-Plex 100 system (Bio-Rad). Cytokine levels were determined by comparison with standard curves generated from murine recombinant cytokines and analyzed using Bio-Plex Manager software (Bio-Rad).

6.3.8. Assay of KC in BAL fluid

Levels of KC in BAL fluid were assayed using Quantikine mouse KC assay kit (R & D systems) according to manufacturer's instructions. Briefly, in a 96-well microtiter plate 50 µl of assay diluent, standards and samples were added per well. The plate was covered with adhesive plate sealer and incubated for 2 hours at room temperature. The plate was washed with wash buffer and 100 µl of mouse KC conjugate was added and incubated for 2 hours at room temperature. After washing, 100 µl of substrate solution was added and incubated for 30 minutes. The reaction was stopped by adding 100 µl stop solution and optical density was measured at 450 nm wavelength. KC levels were determined by comparison with a standard curve generated from murine recombinant KC.

6.3.9. Lung histology

After the mice were euthanized, the left lung was excised, fixed with 4% paraformaldehyde overnight at 4°C, processed by routine histological technique and embedded in paraffin. Five µm thick sections were placed on lysine-coated slides and were stained with hematoxylin and eosin.

6.3.10. Blood collection and neutrophil isolation for chemotaxis experiment

Blood samples were collected from clinically healthy adult dogs (n=3) in Veterinary Medicine Complex, Western College of Veterinary Medicine, University of Saskatchewan. The blood was diluted by mixing 1.5 ml of blood with 0.5 ml of normal saline just before the neutrophil isolation.

Neutrophils were isolated as per the previously described method with few modifications (Comazzi, Paltrinieri et al. 2001). Briefly, equal volumes of isotonic percoll solutions of different densities (1.096 and 1.072) were overlaid on each other. Blood was carefully layered over percoll gradients and centrifuged at 400 g for 20 min. A band of PMNs formed between two percoll gradients was collected and contaminating erythrocytes were lysed by adding 5 ml of sterile distilled water for 1 min and isotonicity restored by adding 2.5 ml of 0.3 mol/L NaCl in 0.0132 mol/l phosphate buffer pH 7.2. The cell suspension was centrifuged at 400 X g for 12 min and washed twice with phosphate buffer saline. Neutrophils were resuspended in 1ml RPMI 1640 media and counted to determine the cell number and the cell viability was assessed by trypan blue exclusion. The cell suspension was cytocentrifuged, stained with May-Grunwald Giemsa and differential cell count performed to determine the purity of cells. The purity of neutrophils was always >95%.

6.3.11. Chemotaxis assay

Canine neutrophil chemotaxis assay was performed as per the previously described method (Comazzi, Paltrinieri et al. 2001). The assay was done using a 12-well modified Boyden chamber (Neuroprobe Inc.) and cellulose-nitrate filters (thickness, 150 mm; pores, 3 mm). Recombinant human interleukin-8 ((Peprotek) at the concentration of 25 ng/ml was put in lower wells as the chemoattractant. The upper wells were filled with 120 µl of neutrophil suspension (2×10^6 per ml) with only media or with LPS (1 µg/ml) alone or LPS plus retinoic acid (5 µM). Samples were incubated at 37°C in 5% CO₂ for 1 hour. At the end of the incubation period, the membrane was washed, and stained with hematoxylin and eosin. The stained membrane was mounted on a glass slide and cells were counted in 5 random fields under light microscopy at 400X magnification. The experiment was repeated thrice.

6.3.12. Statistical analysis

Data are presented as mean \pm SEM and were analysed by one-way ANOVA. Groups were compared by Tukey's post hoc test and a P value of ≤ 0.05 was considered as statistically significant.

6.4. Results

6.4.1. Total and differential cell count in bronchoalveolar lavage fluid

Lipopolysaccharide (LPS) administration induced a significant increase in total cell count in BAL fluid at all the time points examined in this study compared to saline treatment groups (Figure 6.1 shows data for 6 hours time point). Pretreatment with retinoic acid 30 minutes before LPS administration significantly reduced the total cell count at only 6 hours (Figure 6.1). Retinoic acid did not produce significant differences at 12 and 24 hour after LPS treatment (data not shown). The differential cell count revealed that the percentage of macrophages significantly decreased and neutrophils were significantly increased at 6 hours following LPS treatment (Figure 6.2B). When the animals were pre-treated with retinoic acid before LPS administration the percentage of neutrophils was significantly decreased (Figure 6.2B).

6.4.2. Lung myeloperoxidase

The myeloperoxidase (MPO) is considered as a marker of neutrophil infiltration into lungs. Therefore, immunohistochemical staining of MPO was performed to determine the effect of retinoic acid on LPS-induced neutrophil infiltration into lungs. In the LPS-treated animals, numbers of MPO-positive cells were increased significantly at 6 hours compared to saline treated animals (Figure 6.3 & 6.4). Retinoic acid pre-treatment resulted in significant reduction of MPO-positive cells (Figure 6.4 & 6.4) at 6 hours following LPS treatment.

6.4.3. Protein levels in bronchoalveolar lavage (BAL) fluid

The protein content in BAL fluid indicative of increased vascular permeability was significantly increased at all three time points in LPS treated groups compared to saline treatment (Figure 6.5 shows data for 6 hours time point). Similar to total cell count, protein content was significantly reduced in animals pretreated with retinoic acid before LPS instillation only at 6 hours (Figure 6.5). At 12 and 24 hours time points there were no significant differences in BALF protein content between LPS treated groups and retinoic acid plus LPS treated groups (data not shown).

6.4.4. Cytokine and chemokine levels in BAL fluid

Since activated immune and other cells such as epithelial and endothelial cells secrete inflammatory cytokines, concentrations of TNF- α , IL-1 β , and IL-10 were measured in BAL fluid. Intranasal LPS treatment induced a significant increase in the levels of TNF- α in BAL fluid compared to saline-treated animals (Figure 6.6). Unlike total cell count and protein content, retinoic acid pretreatment did not inhibit concentrations of cytokines in BAL fluid (Figure 6.6-6.8). There were no significant differences levels of IL-1 β and IL-10 in BALF between different treatment groups. The levels of chemokines such as KC levels were significantly increased in BAL fluid of LPS treated groups compared to saline-treated animals (Figure 6.9). However, retinoic acid pre-treatment did not inhibit concentrations of KC (Figure 6.9).

6.4.5. Histological examination

Histological examination of lungs from all treatment groups was performed to assess the lung injury. Compared to the normal architecture of lungs from control mice (Figure 6.10A), intranasal LPS instillation produced characteristic features of lung inflammation such as infiltration of inflammatory cells in alveoli and thickening of alveolar wall (Figure 6.10B). Pre-treatment with retinoic acid before LPS instillation slightly diminished these histological alterations at 6 hours

(Figure 6.10C). Perivascular accumulation of inflammatory cells observed in lungs of LPS-treated mice (Figure 6.10B) was highly diminished with pre-treatment of retinoic acid (Figure 6.10C).

6.4.6. Chemotaxis assay

To further study the effects of retinoic acid on neutrophil functions, chemotaxis assay was performed. Activation of neutrophils with LPS resulted in significantly increased number of neutrophils migrated toward human interleukin-8 (Figure 6.11). However addition of retinoic acid in the presence of LPS did not affect the LPS-induced neutrophil chemotaxis.

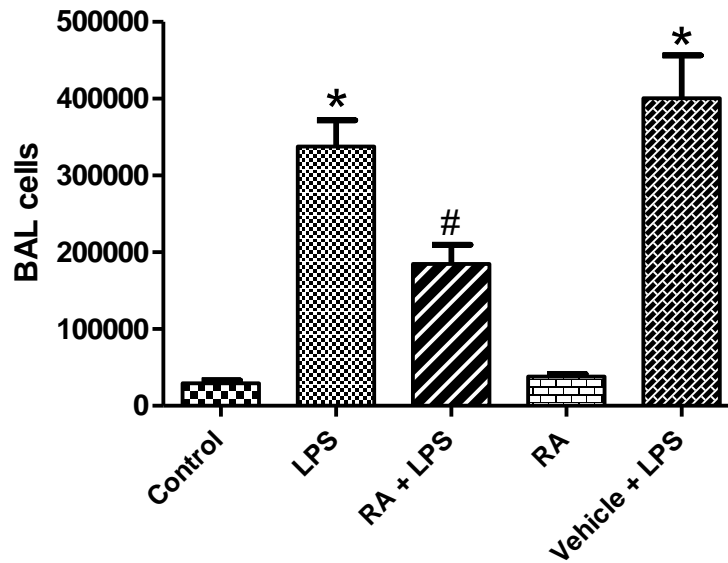


Figure 6.1. Effect of retinoic acid pre-treatment on bronchoalveolar lavage fluid cell count in LPS-challenged mice. Mice were given retinoic acid (10 mg/kg) intraperitoneally, 30 minutes prior to intranasal instillation of LPS. Bronchoalveolar lavage was done at 6 h following LPS treatment and total cell count was done. Each bar represented the mean \pm SEM of 5 mice per group. # $P < 0.05$ vs. control group; * $P < 0.05$ vs. LPS alone group.

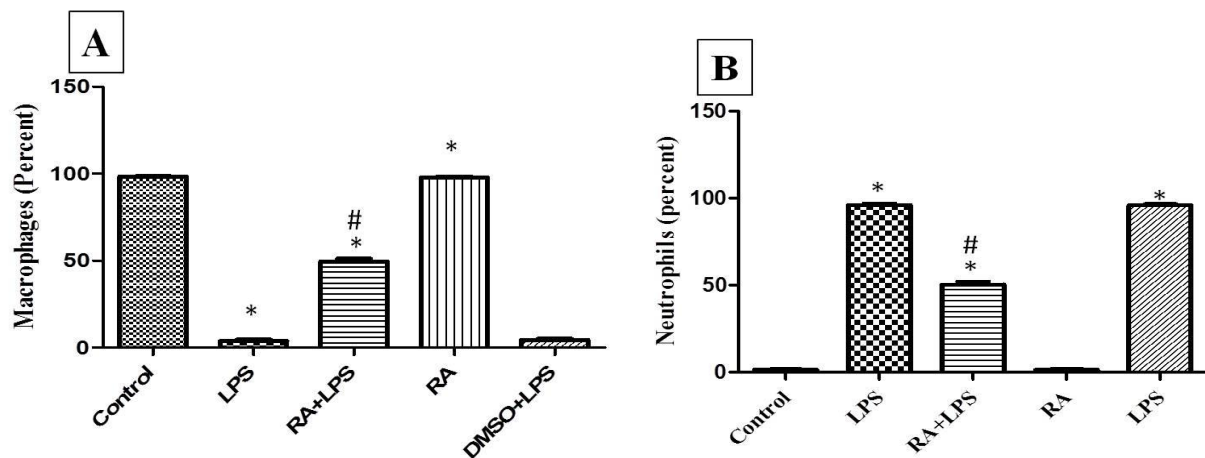


Figure 6.2. Effects of retinoic acid pre-treatment on the differential cell counts in BAL fluid cytopspins of LPS-treated mice. Mice were given retinoic acid (10 mg/kg) intraperitoneally, 30 minutes prior to intranasal instillation of LPS. The percentage of macrophages (A) and neutrophils (B) present in BAL fluid cytopspins at 6 hours following LPS treatment. Data are expressed as mean \pm SE of five mice per group. * $P < 0.05$ versus control and RA, # $P < 0.05$ versus LPS.

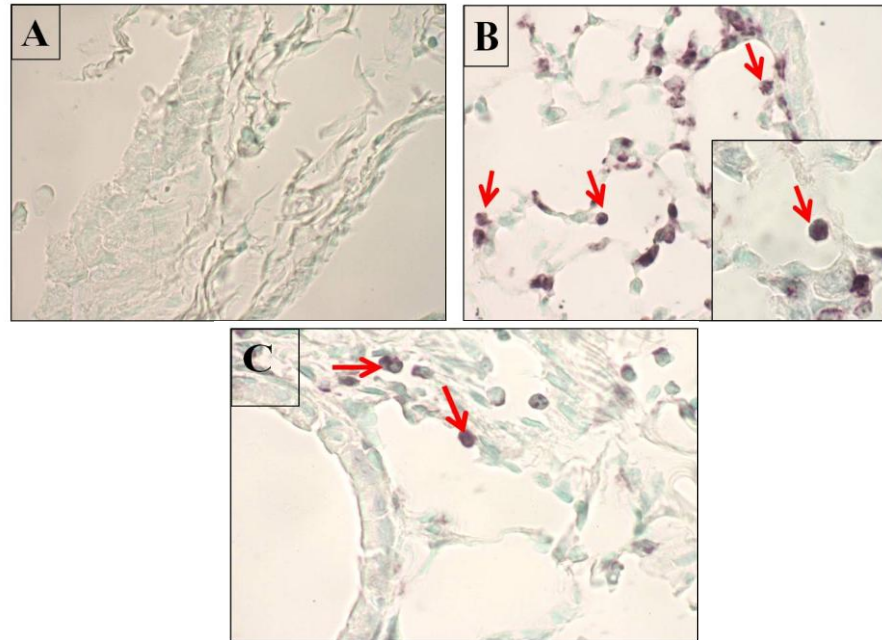


Figure 6.3. Representative pictures of immunohistochemistry for MPO in mouse lung. Saline treated mice (A) with no MPO staining. LPS treatment (B) resulted in large number of MPO-positive cells (arrows) and pre-treatment with retinoic acid (C) resulted in the reduction of MPO-positive cells.

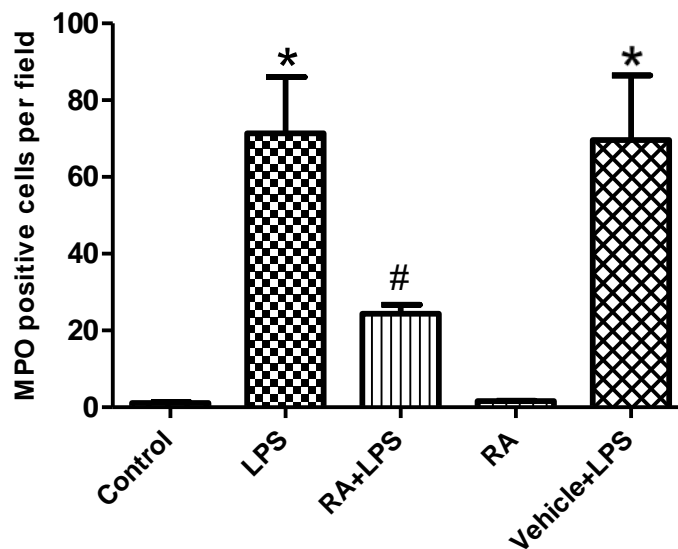


Figure 6.4. Effects of retinoic acid pre-treatment on MPO-positive cells in lungs of LPS-treated mice. Mice were given retinoic acid (10 mg/kg) by i.p. route 30 minutes prior to intranasal instillation of LPS. MPO positive cells were counted in five randomly selected fields from three mice per group. Data are represented as mean \pm SE of three animals per group. * $P < 0.05$ versus control and RA groups, # $P < 0.05$ versus LPS.

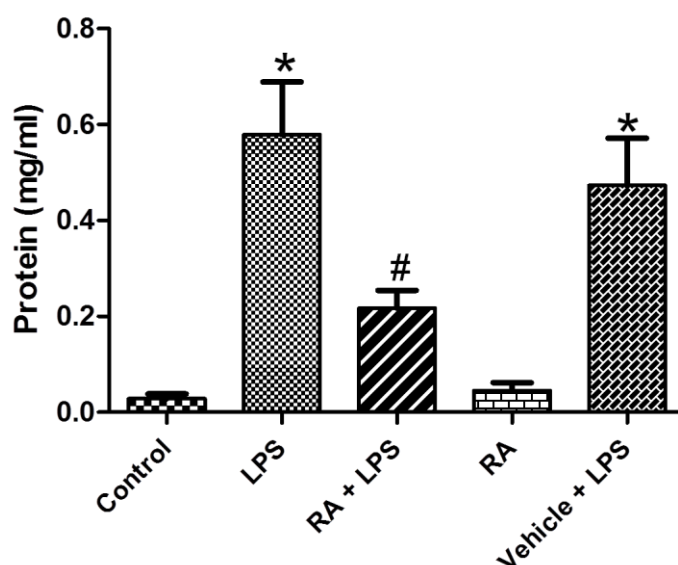


Figure 6.5. Effect of retinoic acid pre-treatment on protein content in bronchoalveolar lavage fluid in LPS-challenged mice. Mice were given retinoic acid (10 mg/kg) intraperitoneally, 30 minutes prior to intranasal instillation of LPS. Bronchoalveolar lavage fluid was collected at 6 h following LPS treatment and protein concentration was measured. Each bar represented the mean \pm SEM of 5 mice per group. * $P < 0.05$ vs. control and RA groups; # $P < 0.05$ vs. LPS alone group.

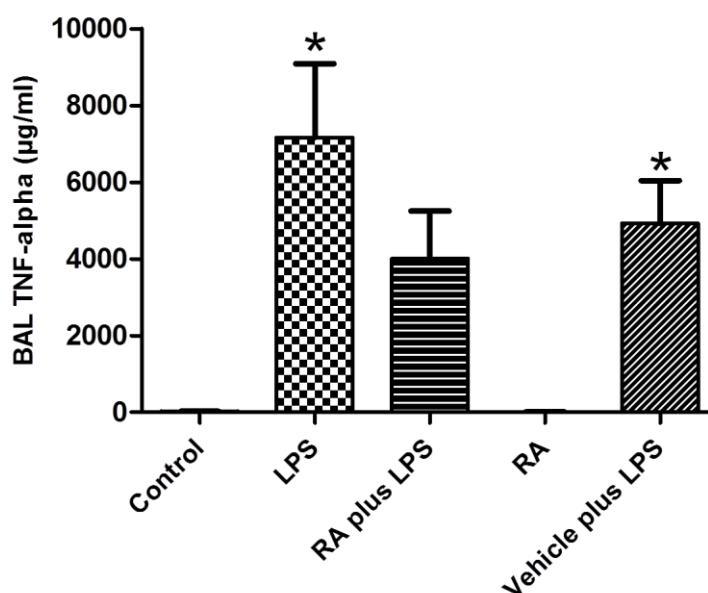


Figure 6.6. Effect of retinoic acid pre-treatment on TNF- α levels in bronchoalveolar lavage fluid in LPS-challenged mice. Mice were given retinoic acid (10 mg/kg) intraperitoneally, 30 minutes prior to intranasal instillation of LPS. Bronchoalveolar lavage fluid was collected at 6 h following LPS treatment and cytokine concentrations were measured by bioplex cytokine assay method. Each bar represented the mean \pm SEM of 5 mice per group. * $P < 0.05$ vs. control and RA group.

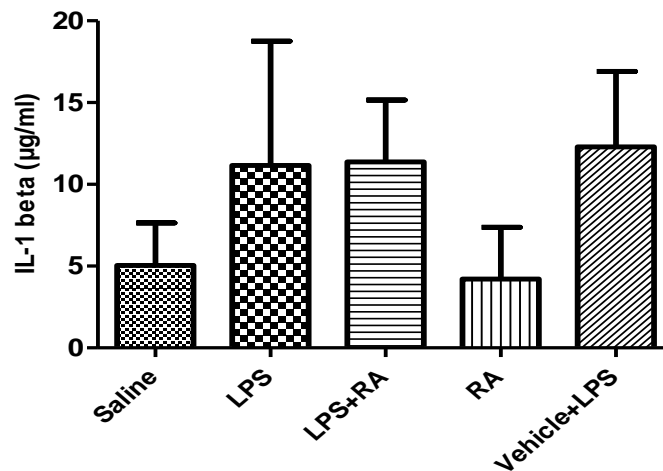


Figure 6.7. Effect of retinoic acid pre-treatment on IL-1 β levels in bronchoalveolar lavage fluid in LPS-challenged mice. Mice were given retinoic acid (10 mg/kg) intraperitoneally, 30 minutes prior to intranasal instillation of LPS. Bronchoalveolar lavage fluid was collected at 6 h following LPS treatment and cytokine concentrations were measured by bioplex cytokine assay method. Each bar represented the mean \pm SEM of 5 mice per group.

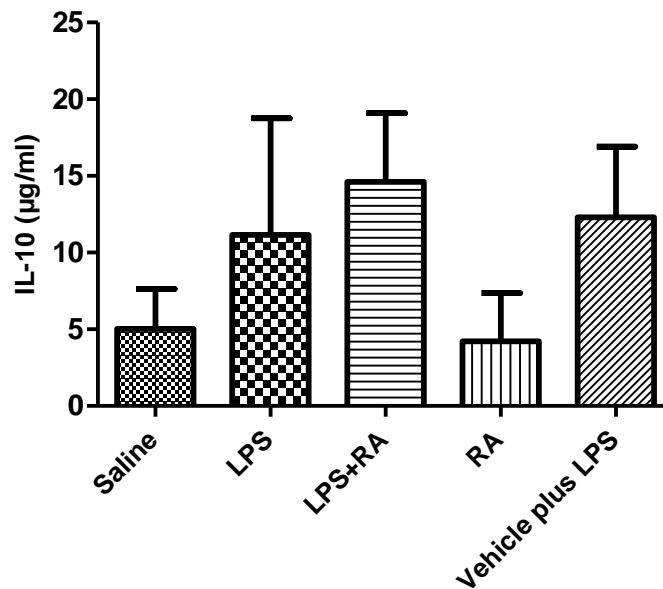


Figure 6.8. Effect of retinoic acid pre-treatment on IL-10 levels in bronchoalveolar lavage fluid in LPS-challenged mice. Mice were given retinoic acid (10 mg/kg) intraperitoneally, 30 minutes prior to intranasal instillation of LPS. Bronchoalveolar lavage fluid was collected at 6 h following LPS treatment and cytokine concentrations were measured by bioplex cytokine assay method. Each bar represented the mean \pm SEM of 5 mice per group.

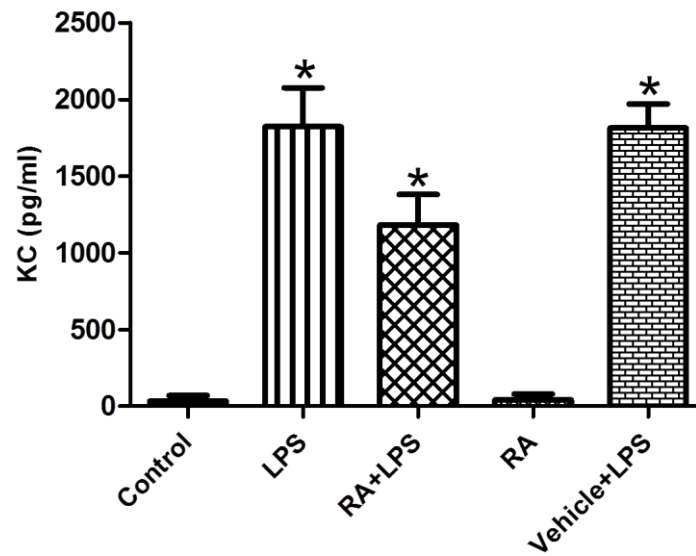


Figure 6.9. Effect of retinoic acid pre-treatment on KC levels in bronchoalveolar lavage fluid in LPS-challenged mice. Mice were given retinoic acid (10 mg/kg) intraperitoneally, 30 minutes prior to intranasal instillation of LPS. Bronchoalveolar lavage fluid was collected at 6 h following LPS treatment and cytokine concentrations were measured by bioplex cytokine assay method. Each bar represented the mean \pm SEM of 5 mice per group. * $P < 0.05$ vs. control and RA groups.

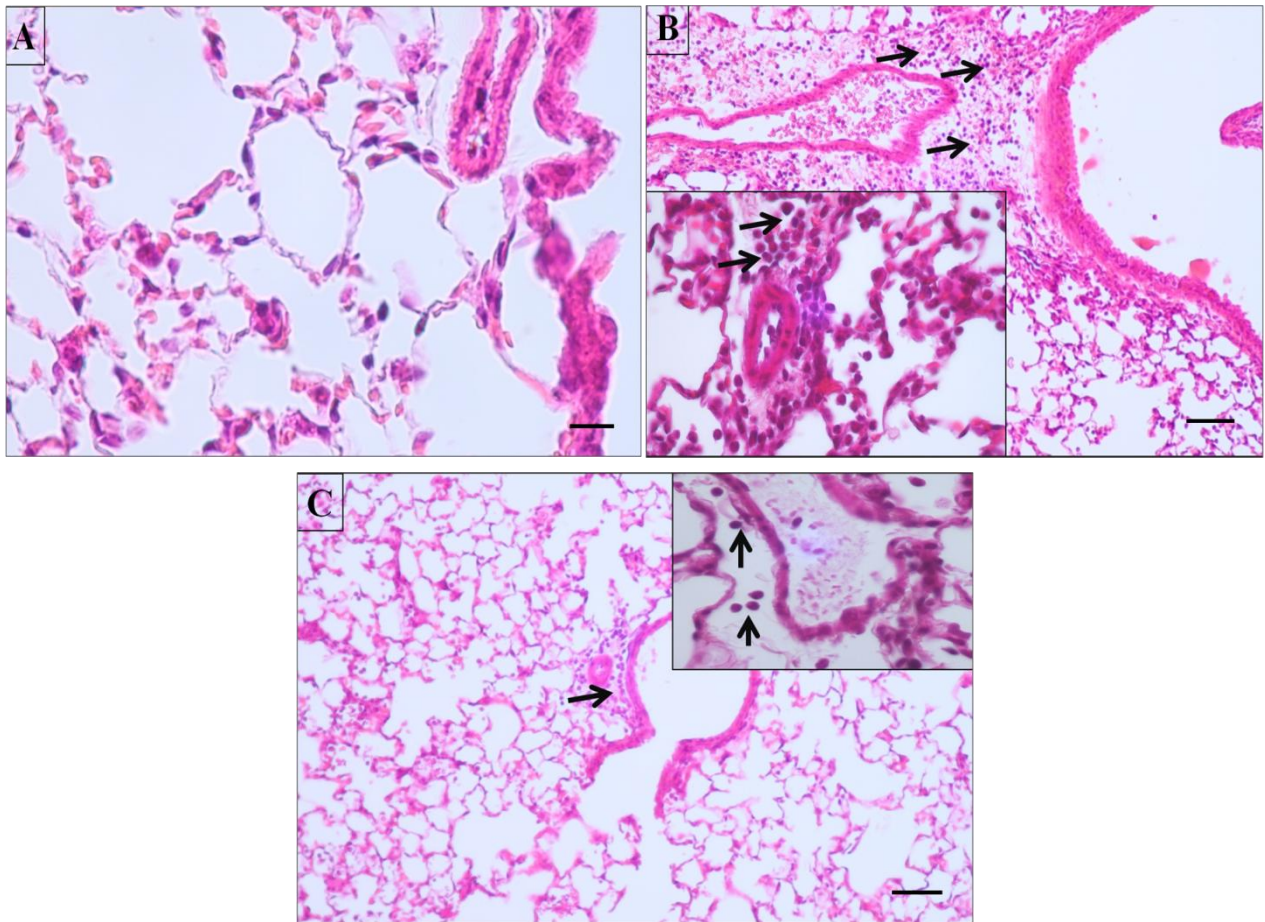


Figure 6.10. Effects of retinoic acid pre-treatment on histological changes in lungs of LPS-treated mice. Mice were given retinoic acid (10 mg/kg) intraperitoneally, 30 minutes prior to intranasal instillation of LPS. H & E stained lung sections from saline-treated mice (A) showing normal architecture of lungs, while in LPS-treated mice (B) showing massive infiltration of inflammatory cells (arrows). In retinoic acid pre-treatment plus LPS treated mice there is a reduction in infiltration of inflammatory cells(C). Bar size: 4 μ M.

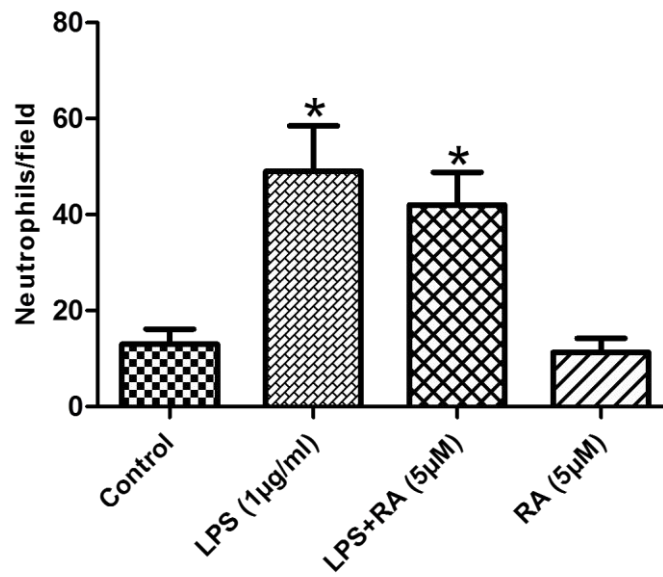


Figure 6.11: Effect of retinoic acid on canine neutrophil chemotaxis. Neutrophil migration was determined by counting the number of neutrophils migrated towards recombinant human interleukin-8 after 60 minutes of incubation with LPS alone or LPS plus retinoic acid. Results represent mean \pm SEM of three independent experiments. * $P < 0.05$ versus control and RA groups.

6.5. Discussion:

Acute lung injury is one of the major causes of respiratory failure and it is associated with high morbidity (Ware and Matthay 2000). It is characterized by diffuse damage to pulmonary parenchyma through a hyperinflammatory response mediated mainly by neutrophils (Grommes and Soehnlein 2011). However, alveolar macrophages and endothelial cells also contribute to pathogenesis of acute lung injury through regulation of neutrophil recruitment and activation (Rubins 2003; Maniatis, Kotanidou et al. 2008). One of the therapeutic strategies to reduce lung injury is to suppress the inflammation-induced tissue injury. Retinoids are vitamin A and chemically related compounds that have been shown to possess antiinflammatory properties. Previous studies have established that retinoids possess anti-inflammatory properties through their suppressive effects on transcription factors such as NF- κ B and activator protein-1 which regulate the gene transcription of inflammatory cytokines (Austena, Carlsen et al. 2009). They have also been shown to inhibit proinflammatory functions of neutrophils and macrophages (Varani, Jones et al. 1991; Mathew and Sharma 2000). Based on these previous reports, I hypothesised that retinoic acid treatment ameliorates endotoxin-induced acute lung inflammation. In the present study, I investigated the effect of retinoic acid pre-treatment on lung inflammation using a murine model of endotoxin-induced acute lung injury. LPS-induced acute lung inflammation model is a reproducible animal model of acute lung injury characterized by marked neutrophilia and damage to lung parenchyma (Matute-Bello, Frevert et al. 2008). In the present study, I also found a marked increase in cell count in BAL fluid. In addition there was a significant increase in protein concentration in BAL fluid. LPS causes endothelial injury with subsequent alteration of permeability, resulting in leakage of protein rich fluid from circulation (Wang le, Patel et al. 2002). My results are in agreement with previous reports showing induction of lung inflammation through LPS treatment. There was also a significant

increase in the levels of cytokines such as TNF- α in BAL fluid which is consistent with previously reported data (Matute-Bello, Winn et al. 2004; Puljic, Benediktus et al. 2007). These data validated the mouse model of endotoxin-induced acute lung injury.

Retinoic acid pre-treatment did alter some features of LPS-induced lung inflammation. Pre-treatment with retinoic acid significantly decreased total cell counts, percentage of neutrophils and protein levels in BAL fluid at 6 hours but not 12 or 24 hours after LPS-treatment. The increased cell counts and protein levels in BAL indicate compromised vascular barrier in LPS-treated animals (Penn and Chisolm 1991). The inhibition of cell recruitment and edema formation by retinoic acid suggests strengthening of lung vascular barrier. Moreover the protein levels were also significantly decreased in animals pretreated with retinoic acid. There are previous data showing decreased neutrophil recruitment to lung following retinoic acid treatment in different models of lung inflammation (Torii, Miyake et al. 2004). Retinoic acid is known to maintain and repair epithelial/endothelial integrity by promoting barrier function of tight junctions. Promoting barrier function is associated with upregulation of tight junction associated genes such as occludin, claudin-1, claudin-4, and zonula occludens-1 (Osanai, Nishikiori et al. 2007). Retinoic acid also inhibits platelet tissue factor and increases production of tissue plasminogen activator by endothelial cells (Marchetti, Vignoli et al. 2003). There could be direct effects of retinoic acid on neutrophils to deactivate them and to inhibit their migration in response to chemokines because retinoic acids can suppress NF- κ B activation (Kuenzli, Tran et al. 2004). The β 2 integrins play important role in recruitment of neutrophils to inflamed lungs and retinoic acid treatment has been shown to suppress the expression of β 2 integrins in leukocytic cell line and human monocytes (Sjogren, Stendahl et al. 2000; Babina and Henz 2003). The combined effects of retinoic acid on vascular permeability and cell migration suggest

that the effects were through strengthening of endothelial and epithelial barrier in the lung. Nevertheless, retinoic acid suppresses migration of neutrophils and edema formation in lungs of LPS-treated animals.

I also used an anti-myeloperoxidase antibody for in situ localization of neutrophils in intact lungs from all the treatment groups. This was done to complement and strengthen the neutrophil migration data obtained through analyses of BAL. Neutrophils in significant numbers were present in the septum and perivascular areas in the LPS-treated lungs. In contrast, the numbers were significantly decreased in LPS-treated mice pre-treated with retinoic acid. Singh and colleagues have reported the importance of perivascular compartment in inflammatory cell recruitment (Singh, Shinagawa et al. 2005; Tschernig, Janardhan et al. 2008). Reduced accumulation of neutrophils in the perivascular compartment shows that neutrophils in retinoic acid treated mice didn't cross the endothelial barrier.

Lung inflammation is regulated by multiple inflammatory cytokines such as TNF, IL-1 β and IL-10 and chemokines such as KC. The outcome of an inflammatory response depends on the balance between pro-inflammatory and anti-inflammatory molecules. In this context, while TNF and IL-1 are pro-inflammatory cytokines and induce expression of adhesion molecules and activate endothelial and other cells, IL-10 is an anti-inflammatory regulatory cytokine. KC is a chemokine which induces neutrophil recruitment into lungs. Intranasal instillation of LPS activates airway epithelium and alveolar macrophages leading to production of pro-inflammatory cytokines. Retinoic acid did not affect the concentrations of cytokines and chemokines in BAL fluid of LPS-treated animals. The lack of effect on cytokine and chemokine concentrations is intriguing considering that retinoic acid suppresses NF-kB transcription and translocation (Kuenzli, Tran et al. 2004; Austenaa, Carlsen et al. 2009). Although the precise reasons for the lack of cytokine effect of retinoic acid on

NF- κ B were not investigated, there are some possibilities. Since inflammation is a highly dynamic interplay of cytokines and cells, I may have missed the time point of effect of retinoic acid on cytokines. A second possibility could be that lung tissue cytokine levels may have been affected but we did not probe the tissue for cytokine levels. Lastly, it is possible that retinoic acid mainly affects the neutrophils through deactivation to manifest its anti-inflammatory effects.

I also investigated the effects of retinoic acid on chemotaxis of activated canine neutrophils. Activation of neutrophils induced significant chemotaxis of neutrophils towards human interleukin-8; however retinoic acid failed to inhibit neutrophil chemotaxis. Synthetic retinoids have also been shown to inhibit chemotaxis of monocytes and neutrophils (Norris, Osborn et al. 1987). Lack of effect may be due to a species specific effect of retinoic acid and effects of retinoic acid may vary depending on the experimental conditions. The lack of effect of retinoic acid on neutrophil chemotaxis further suggests that retinoic acid may be primarily promoting integrity of endothelial and epithelial barrier. However, further studies are needed to characterize the effects of retinoic acid on neutrophil cytoskeleton and chemotaxis.

There was no inhibitory effect of retinoic acid on BAL cell count and protein levels at 12 and 24 hour time points. We don't know the precise reasons for the transient effect of retinoic acid on LPS-induced acute lung inflammation. However, there are some possibilities. The lack of effect at later time points may be due to shorter half life of retinoic acid in rodent models as supported by previous studies (McPhillips, Kalin et al. 1987; Le Doze, Debruyne et al. 2000). There clearly is a need to undertake further studies on therapeutic actions of retinoic acids through multiple treatments.

In conclusion, I have shown the transient amelioration of endotoxin-induced acute lung injury following pre-treatment with retinoic acid. Although normally people would take vitamin A every day, but a case can be made to treat the animals with retinoic acid after exposing them to

endotoxin. It also would be useful to determine the extent of neutrophil apoptosis in retinoic acid treated LPS-challenged animals. Further studies are needed to study the protective effects of retinoic acid either by altering the delivery or treatment regimen.

CHAPTER 7: GENERAL DISCUSSION

The main focus of my research was to investigate the role of retinoids in acute lung inflammation and neutrophil biology. Since retinoid receptors mediate the effects of retinoids, I first determined the expression pattern of retinoid receptors in different species including mice and human lungs. Because neutrophil apoptosis plays a key role in pathogenesis of acute lung inflammation, I then determined the effects of retinoic acid on spontaneous apoptosis of resting and activated canine neutrophils. Having determined expression pattern of retinoid receptors and effect of retinoic acid on neutrophil apoptosis, I finally investigated the effects of retinoic acid on endotoxin-induced acute lung inflammation.

I determined the spatial expression of retinoid receptors normal lungs from mouse, human, cattle, dog, and pig with the use of multiple techniques. The focus of the immunohistochemical method was to characterize their expression pattern in specific pulmonary cell types which play important roles in lung physiology. There was no information available on the expression pattern of retinoid receptors in any of domestic animal species. First, I used western blot to determine the expression of retinoid receptor subtypes in pig lung, and the data showed expression of all six subtypes. Secondly, I used immunohistochemistry to determine the expression of retinoid receptors in different pulmonary cell types. The retinoid receptors subtypes were found in bronchial epithelium, vascular endothelium, alveolar/septal macrophages, and alveolar epithelium. The data also indicated differential expression of retinoid receptors in lungs of cattle, dog, and pig. Presence of several subtypes and different heterodimers combinations lead to diverse effects of retinoids. Differential expression of retinoid receptors may indicate distinct physiological function and this knowledge helps in developing a receptor selective therapeutic strategies. Differential expression pattern may lead to diverse effects

depending on the receptor subtype activated and heterodimers formed. Gene expression regulation by retinoid receptors depend on their spatio-temporal expression. For example, RAR γ is expressed in high levels in skin and the expression levels correlate with predominant effects of RAR γ ligand in skin (Fisher, Talwar et al. 1994; Mangelsdorf 1994; Standeven, Teng et al. 1997). Differential effects are supported by distinct symptoms of vitamin A deficiency with knockouts of different receptors (Kastner, Mark et al. 1995; Chiang, Misner et al. 1998). However, the implications of such knockouts on immune system and lung inflammation haven't been studied. Furthermore, each retinoid receptor subtype can exert distinct effect within the same cell (Nagpal, Saunders et al. 1992; Nagpal, Friant et al. 1993). Data on spatial expression of retinoid receptor subtypes opens up the possibility of use of subtype-specific retinoids with reduced side effects.

The use of immune-electron microscopy declined significantly in the 90s with the emergence of confocal microscopy. However, without the use of immuno-electron microscopy, there lies a danger of missing lots of information on subcellular localization of proteins and other molecules. To avoid that, I used immuno-electron microscopy to show sub-cellular localization of retinoid receptors in these species. Immuno-electron microscopy showed expression of retinoid receptors in the nucleus. In addition, electron microscopy also helped to identify cell types such as pulmonary intravascular macrophages, monocytes, and neutrophils based on the morphology and expression of retinoid receptor subtypes in these cell types. Electron microscopy also revealed cytoplasmic localization of retinoid receptors, which belong to nuclear receptor superfamily, but have been localized in cytoplasm in certain phases of cell cycles including inflammation (Katagiri, Takeda et al. 2000; Mey, Schrage et al. 2007). Previous studies have also shown intracytoplasmic localization of RAR γ HeLa cells (Han, Zhou et al.

2009). Relocalization of RXR α into cytoplasm results in the suppression of RXR α -mediated gene transcription (Ghose, Zimmerman et al. 2004). The data indicate cycling of retinoid receptors between nucleus and cytoplasm, which may be an on and off switch for gene transcription.

Receptor expression is a dynamic process, and the expression is affected in activated cells. Using western blot and immunohistology, I observed that retinoid receptor expression was modified, such as increased expression of RXR α in inflamed mouse lungs. While light microscopy localized the expression of various receptor subtypes in the bronchial epithelium, vascular endothelium, alveolar septa, and alveolar/septal macrophages, immuno-electron microscopy detected the receptors in the nucleus of various cells. Expression of RAR α , RAR β , and RAR γ have been shown in different stages of rat lung development by quantitative methods such as real time PCR and northern hybridization (Grummer, Thet et al. 1994).

Immunolocalization of retinoid receptor subtypes has been done in human fetal lungs to show their role in human lung development (Kimura, Suzuki et al. 2002). Expression of various RAR isoforms has also been reported in postnatal mouse lung (Hind, Corcoran et al. 2002). Using in situ hybridization authors found expression of all three RAR subtypes in bronchial epithelium. In our study we did not find expression of RAR α in bronchial epithelium. Discrepancy could be due to age related changes in the expression pattern of retinoid receptors. There has been only one study to look at the expression of retinoid receptors in an inflammatory disease. Expression in airway epithelium has been shown in chronic allergic inflammatory disease such as asthma (Druihe, Zahm et al. 2008). Epithelial expression of RAR γ , RXR α , and RXR γ has been reported to be upregulated in asthmatic patients compared to healthy controls. In addition, expression of retinoid receptors was positively correlated with morphologically intact epithelium which

indicates their possible role in epithelial repair in inflammation. Treatment of airway epithelial cells with retinoids leads to increased expression of transforming growth factor (TGF)- β transcripts indicating the role of retinoids in epithelial repair. In the present study, we used murine model of acute lung inflammation and data indicated upregulation of RXR α in bronchial epithelium. Increased expression of RXR α indicates its possible role in epithelial repair in inflammation. Retinoic acid induces proliferation of alveolar epithelial cells and promotes epithelial repair (Belloni, Garvin et al. 2000). Retinoids promote regeneration of alveolar epithelium injured by elastase (Massaro and Massaro 1997). Airway epithelium plays a critical role in regulating lung inflammation. It has been shown to regulate inflammatory response and histopathological changes in asthma (Erjefalt 2010). Airway epithelial cells release and respond to inflammatory mediators secreted by other cells (Cohn and Adler 1992; Khair, Davies et al. 1996). Retinoid X receptor inhibits leukotriene signaling pathways resulting in resolution of inflammation (Kalsotra, Du et al. 2008). Agonists of retinoid receptors have also been shown to suppress inflammation dependent survival of breast cancer stem cells (Papi, Guarnieri et al. 2012). Contrast to these reports, RXR α has been shown to regulate transcription of chemokines in macrophages (Nunez, Alameda et al. 2010). Although we observed upregulation of RXR α in murine model we did not see differences between normal and septic human lungs for any of the retinoid receptor subtype. Lack of difference is explained by the fact that expression of these receptors may be time dependent and samples from septic cases were collected after the death of patients.

Neutrophils play a central role in pulmonary defence but are also credited with causing significant damage to the inflamed lungs. It has been proposed that one of the ways to modulate neutrophil behaviour is to shorten their lifespan by hastening their spontaneous apoptosis. My *in*

vitro data show pro-apoptotic effects of retinoic acid on activated neutrophils. One of the strengths of my data is the use of multiple methods to detect apoptosis because apoptosis is a complex process and one single method may not be robust enough. The combined use of light and electron microscopy, TUNEL and flow cytometry provided complimentary data. My data from neutrophils are similar to previous reports of pro-apoptotic effects of retinoic acid in a number of cell lines. Retinoic acid induces apoptosis of T lymphocytes through RAR γ (Szondy, Reichert et al. 1998). However, this is the first study to investigate the effect of retinoic acid on apoptosis of activated neutrophils. Initiation of neutrophil apoptosis is associated with impairment of neutrophil proinflammatory functions (Whyte, Meagher et al. 1993). It also leads to recognition by macrophages and phagocytosis of apoptotic neutrophils by macrophages results in inhibition of proinflammatory cytokines production and increases secretion of anti-inflammatory cytokines such as IL-10 and TGF β (Fadok, Bratton et al. 1998). The data from my study indicate that retinoic acid might decrease the response of neutrophils to pathogen-derived factors, such as LPS or pro-inflammatory cytokines secreted by immune cells. The pro-apoptotic effects of retinoic on spontaneous apoptosis of activated neutrophils may hasten the elimination of activated neutrophils from the inflamed sites. Therefore, these effects have implications for minimizing the tissue damage and promoting the resolution of inflammation. Retinoic acid induces apoptosis by activating both intrinsic and extrinsic pathways (Altucci, Rossin et al. 2001; Niu, Menard et al. 2001). The data didn't support my hypothesis that retinoic acid may be affecting apoptosis by increasing caspase-3 expression in neutrophils. This leaves room for further studies to determine precise mechanisms of action through which retinoic acid induces apoptosis in activated neutrophils. However, multiple evidences of apoptosis in retinoic acid-

treated neutrophils indicate a potential therapeutic role for retinoids in neutrophil-dominated acute inflammatory diseases.

My data show transient inhibition of acute lung inflammation by retinoic acid in a well established mouse model of acute lung injury. While retinoic acid inhibited total cell numbers, neutrophil percentages and protein concentration in BAL at 6 hours, the effects were not apparent at 12 and 24 hours. These results are in agreement with previous results which showed decreased neutrophilia in response to different stimuli (Hisada, Adcock et al. 1999; Torii, Miyake et al. 2004). Retinoic acid may have inhibited the neutrophil migration through multiple effects including strengthening of endothelial barrier, silencing of activated neutrophils, anti-thrombotic effects on endothelium, and reduced expression of vascular cell adhesion molecule-1 (Gille, Paxton et al. 1997; Marchetti, Vignoli et al. 2003). Retinoic acid reduces production of reactive oxygen species by neutrophils and pre-incubation of endothelial cells with retinoic acid prevents injury from activated neutrophils (Varani, Jones et al. 1991). My *in vitro* showing lack of effects of retinoic acid on neutrophil chemotaxis points to primary beneficial effects through strengthening of endothelial barrier and reduced thrombotic potential of the endothelium. Based on these previous findings, it is reasonable to speculate that retinoic acid could exert its effects through its inhibitory effects both on neutrophils and endothelial cells.

Acute lung injury is a highly complex and cascading process. While neutrophils dominate early phase of the lung inflammation induced with a single intranasal challenge with LPS, the monocytes and macrophages dominate the later parts of the inflammation. The lack of effect at later time points may be due to the relatively shorter half life of retinoic acid in rodent models as reported by previous studies (McPhillips, Kalin et al. 1987; Le Doze, Debruyne et al. 2000) or lack of effects on cells such as monocytes and macrophages. Therefore, multiple treatments with

retinoic acid or local inhaled delivery of retinoic acid may be potentially more beneficial in acute lung inflammation.

Taken together my experiments have provided information on expression of retinoid receptors, effects of retinoic acid on neutrophil apoptosis and endotoxin-induced acute lung inflammation. Results suggest that there is a distinct expression pattern of retinoid receptors between different species. Further, retinoid receptors are widely expressed in cells important in initiating and regulating lung inflammation such as alveolar macrophages, vascular endothelial cells, and neutrophils. There is a need to characterize the specific role of retinoid receptors in these cell types that would lead to possible therapeutic role of retinoid receptor agonists in inflammatory lung diseases. Retinoic acid also abolished the effects of LPS on spontaneous apoptosis of canine neutrophils. Further studies are required to elucidate molecular mechanisms of retinoic acid-induced neutrophil apoptosis and possible in vivo therapeutic potential through regulation of apoptosis in activated neutrophils. Finally, retinoic acid pre-treatment partially inhibits acute lung inflammation (Figure 7.1). There is a need to design experiments with different treatment regimens to further elucidate the effect of retinoic acid on lung inflammation and molecular mechanisms of those effects.

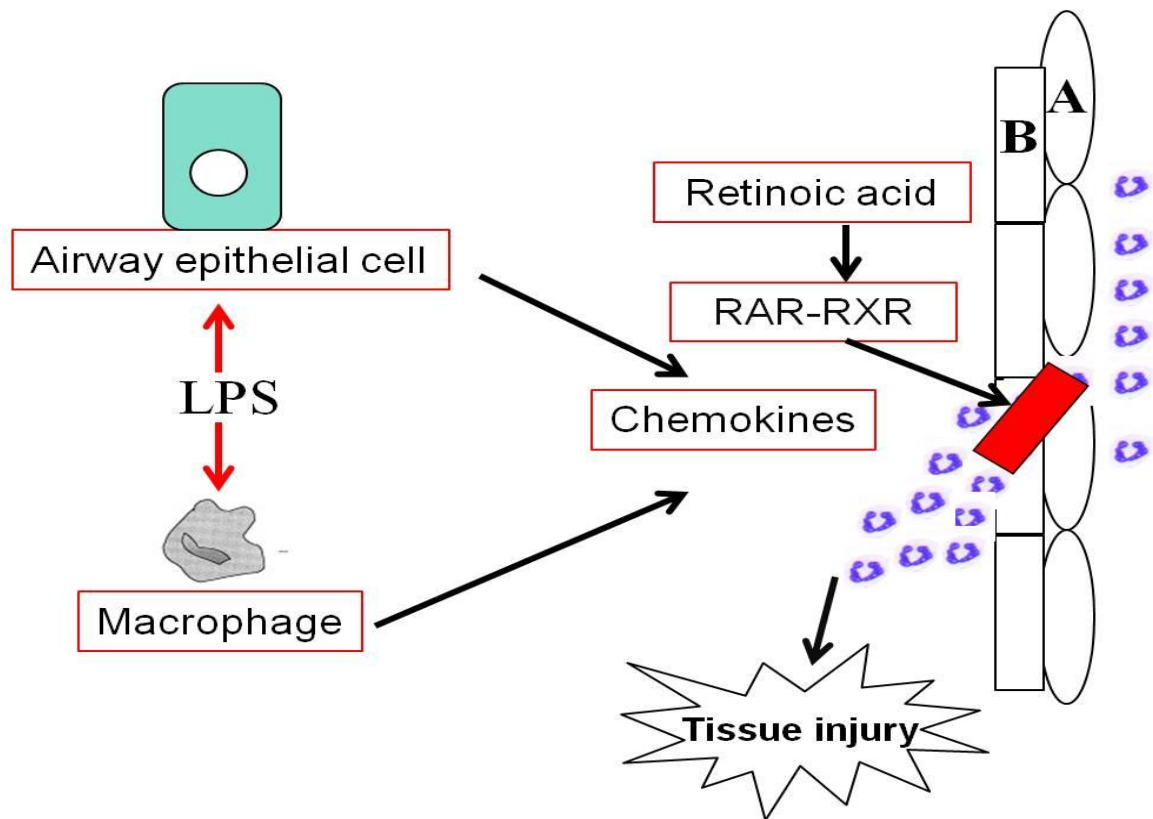


Figure 7.1: Function of retinoic acid in acute lung inflammation. LPS activates airway epithelial cells and macrophages, which secrete chemokines. Chemokines mediate neutrophil recruitment into lungs resulting in tissue injury. Retinoic acid pre-treatment acts through RAR and RXRs on endothelial cells to inhibit neutrophil migration, and tissue injury. A: vascular endothelium. B: alveolar epithelium.

CHAPTER 8: LIMITATIONS OF THE STUDIES

Although data from the above experiments further our knowledge on function of retinoids in relation to pulmonary pathophysiology, there are certain limitations of these studies. There is lack of mRNA data for expression of retinoid receptors in domestic animals species. Also, there are no mechanistic data on retinoic acid-induced neutrophil apoptosis and on the lack of effect of retinoic acid on neutrophil chemotaxis *in vitro*. Furthermore, a comprehensive study on the roles of retinoids on all the aspect of lung inflammation in different models needs to be studied.

CHAPTER 9: FUTURE DIRECTIONS

Further studies are required to examine the role of retinoids in the function of alveolar macrophages, pulmonary intravascular macrophages, neutrophils and endothelial cells in different species. Also, it is necessary to investigate the function of retinoids in pulmonary physiology and pathology in domestic animal species that could lead to new therapeutic approaches in lung diseases. Furthermore, a comprehensive study on mechanism of retinoic acid-induced apoptosis and its implications under *in vivo* conditions need to be studied. In addition, it is necessary to study whether retinoic acid-induced neutrophil apoptosis results in the phagocytosis of apoptotic cells by macrophages. Investigation of function of retinoids in pathogen-induced lung inflammation would help in developing therapeutic strategies to reduce morbidity and mortality because of lung inflammation associated with various disease conditions.

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